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The Localization of the Presumptive Cerebral Regions in the Neural Plate of the Axolotl Larva

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INTRODUCTION

ONE of the most important means of studying the phenomena of induction and determination during the development of the central nervous system consists in microsurgical operations of different kinds on the neural plate of Urodela. Hence it is important to have the neural plate mapped with regard to the prospective significance of its different parts, the 'materielle Anlagen' of Mangold (1937).

A detailed mapping of the caudal part of the neural plate has been carried out by Nakamura (1942). Earlier attempts to map the entire presumptive brain are due to His (1893) who, however, used a very crude method, and to Waechter (1953) who examined the ability to differentiate of material from different parts of the neural plate. Investigations of this kind have also been performed by other authors (Mangold, 1933, 1937, 1955; Mangold & v. Woellwarth, 1950; Raven, 1935; Alderman, 1935; v. Aufsess, 1941; ter Horst, 1947) who were, however, not so confident of the interpretation of their results as to synthesize them in a map of prospective areas. The results of microsurgical operations on the neural plate also provide information about the localization of presumptive regions (e.g. v. Aufsess, 1941; Hörstadius & Sellman, 1946; Raunich, 1951; Slàdeček, 1952 and 1955).

More reliable is the vital staining method of Vogt (1925). This method has been used by several students who, however, all aimed at the examination of a limited area, either the eye field (Petersen, 1923; Woerdeman, 1929; Manchot, 1929) or the neural crest (Baker & Graves, 1939; Fautrez, 1942; Hörstadius & Sellman, 1946). There has been special interest in the position of the presumptive eye areas, and in addition to the investigations by vital staining mentioned, other studies have been made by Spemann (1912), Stockard (1913), Fischel (1921), and Bartelmez (1922), the last-mentioned working with human material.

In the investigations reported here the method of vital staining has been employed. The main aim was the projection upon the neural plate of the most important areas of the fully developed brain. This led the author to the problem

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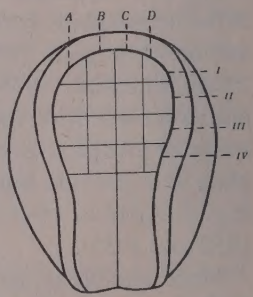
of the limits between presumptive brain, presumptive mesenchyme, and presumptive epidermis, which has previously been dealt with by Woerdeman (1929), Baker & Graves (1939), Fautrez (1942), and Hörstadius & Sellman (1946). Finally, the question as to how far the final determination of the neural plate and of the neural ridge has advanced in the early neurula is discussed.

MATERIAL AND METHODS

The experiments have been carried out on the axolotl, *Siredon mexicanum* Cope. Vital staining was undertaken when the larvae had reached Harrison stage 15. Pieces of agar of suitable size, saturated with the vital stain, were placed when wet upon small glass bridges, in the pattern which it was desired to apply to the larva. On drying, the pieces of agar stuck to the glass, and did not loosen upon subsequent immersion of the bridges in fluid. The initial experiments aimed at putting alternating red and blue marks upon the larva by means of neutral red and Nile-blue sulphate. As Vogt found, however, neutral red tended to disappear from the tissues before the larvae had reached the stage when they were due for fixation. Thus later experiments employed exclusively Nile-blue sulphate which remained in the pigment grains of the cells for at least a fortnight.

The larva was first freed from its membranes. The glass bridge was then laid on it in such a way that the pieces of agar occupied the desired positions upon the neural plate. To facilitate the description of the position of the colour marks, and to permit comparison of the results of different experiments, the plate was plotted into squares as shown in Text-fig. 1. After about 15 minutes staining was stopped. The larvae and their colour spots were drawn with the aid of a drawing apparatus. Subsequent development was carefully observed, and as a rule each larva was drawn several times prior to the closure of the neural tube. It was thus possible to trace the movements of the cells in the neural plate: these observations will be reported in a future paper.



TEXT-FIG. 1. A neurula of *Siredon mexicanum* at stage 15, showing the labelling of the squares used for plotting out the location of vital dye marks on the neural plate.

The stained larvae were reared in tap-water (groundwater from the Uppsala esker). To reduce the risk of infection, sodium sulphadiazine was added to produce a concentration of 0·5 per cent. (Detwiler & Robinson, 1945; Detwiler, Copenhaver, & Robinson, 1947; Copenhaver & Detwiler, 1948). Detwiler *et al.* (1947) recommend an addition of quinine sulphate to prevent attacks upon the larvae by ciliate protozoa. During the later part of the investigation this was done with very good results.

Generally the larvae were fixed when they had attained stage 37–38. A smaller

number, which had been stained upon the transverse ridge and upon the foremost part of the plate, were fixed at various younger stages, from 19 to 28.

Earlier attempts to solve the difficult problem of preserving the vital stain in the cells during embedding and sectioning are described by Vogt (1925). The greatest danger to the stain seems to come from alcoholic dehydration. Lehmann (1929) introduced phosphomolybdic acid as a mordant, and a variation of this method was published by Stone (1932). Experiments with different modifications of Stone's method led to the following treatment which preserves the stain excellently:

1. Fixation in Zenker	1 hour
2. Running water	1 hour
3. 1 per cent. phosphomolybdic acid	1 hour
4. Anhydrous glycerine	overnight (one change)
5. Terpineol + feeble heat (about 40°)	overnight (one change)
6. 40° paraffin	1 hour
7. 52° paraffin	1 hour
8. Embedding in 58° paraffin.	

To make the stain as visible as possible thick sections (15 μ) were used.

Herrick's Fig. 16 in his paper of 1938 served as a pattern for the work of reconstruction during the examination of the sections. The figure shows the inside of the brain of the axolotl larva at the stage called 'early swimming' by Coghill (1929). This roughly corresponds to Harrison stage 37–38. Upon this diagram the stained areas have been marked in the way shown in Text-figs. 2–13, and in certain cases also the origin of the cranial nerves has been indicated. In the diagram Herrick has placed the primordia of the different areas as described by Coghill (1930, paper IX), though with some modifications. Herrick's division of the fore-brain has been discussed by Rudebeck (1945).

Reconstructions of 58 larvae which proved to possess entirely normal brains have been chosen as the basis for the plan of presumptive areas of Text-fig. 15. This method of vital staining cannot be called entirely reliable, since some of the reconstructions show clearly that some diffusion of the stain has taken place. The material was, however, fairly large (generally several colour marks had been applied to each larva in different places), and the results are, with few exceptions, quite unambiguous.

The following is the key to the numerals indicating the different areas, in the diagrams of Text-figs. 2–16:

1. Area olfactoria primitiva
2. Primordium piriforme
3. Primordium hippocampi
4. Septum (primordium)
4*a*. Septum ependymale
5. Nucleus praeopticus, pars anterior
5*a*. Nucleus praeopticus, pars posterior
6. Chiasma ridge
7. Area strio-amygdaloidea
7*a*. Cranial part of pars ventralis thalami

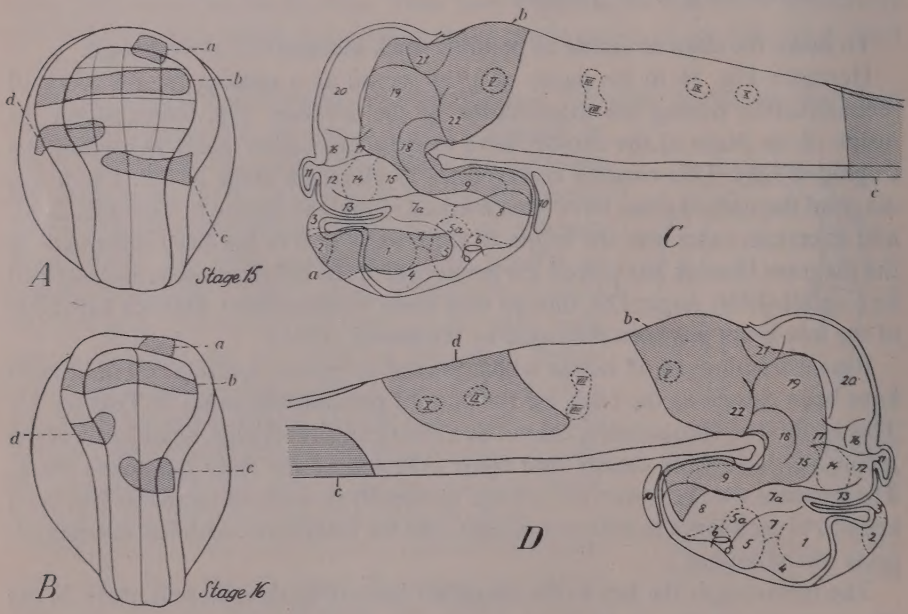
8. Pars ventralis hypothalami
9. Pars dorsalis hypothalami
10. Pars buccalis of the hypophysis
11. Primordium epiphyse
12. Pars intercalaris diencephali
13. Primordium habenulae
14. Pars dorsalis thalami
15. Pars ventralis thalami
16. Commissura posterior (eminentia)
17. Commissura posterior (nucleus)
18. Tuberculum posterius (pedunculus)
19. Tegmentum dorsale
20. Tectum mesencephali
21. Regio cerebellaris
22. Tegmentum isthmi.

A more detailed description of the areas is given by Herrick (1937). The roman figures indicate the place of emergence of the cranial nerves from the brain.

EXPERIMENTAL RESULTS

In this section a number of specimens will be described in detail.

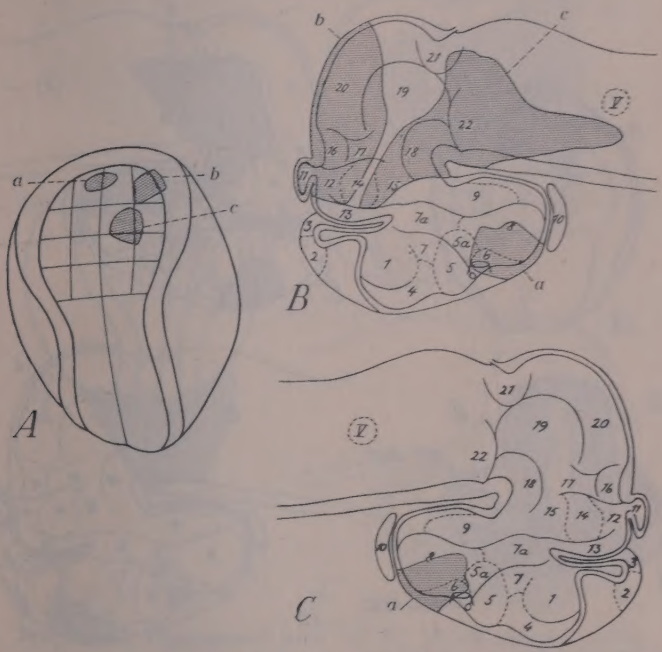
VB 116 (Text-fig. 2). Fixed in stage 37. The colour mark *a*, comprising material of the transverse ridge outside the square I C (see Text-fig. 1) with the



TEXT-FIG. 2. Experimental larva VB 116. *A*, *B*: the position of the marks just after their imposition and at a later stage. *C*, *D*: the position of the marks in the brain at stage 37. The reconstructions are redrawn from Herrick (1937). For key to labels of the different brain areas, see p. 3.

foremost part of I C, was found in the sections within the telencephalon (Text-fig. 2c). The stain had been taken up mostly by the area olfactoria and the

primordial pallium. The brain had not quite reached the stage represented in the reconstruction diagram. The hemispheres, for instance, were only slightly developed, and the plotting of *a* is therefore not quite exact. In the eye, part of the tapetum was stained as well as, ventrally, the transition between the eye-cup and the optic stalk. Part of the nasal placode was stained and part of the epidermis in front of the nostrils. No traces could be found of stained mesenchymal cells which might be derived from the anterior ridge. According to Baker & Graves (1939) a narrow strand of presumptive mesenchyme should exist around the anterior part of the neural ridge, and connect the neural material and the



TEXT-FIG. 3. Experimental larva VB 121. *A*: the position of the marks on the neurula. *B, C*: reconstructions of brain halves at stage 36.

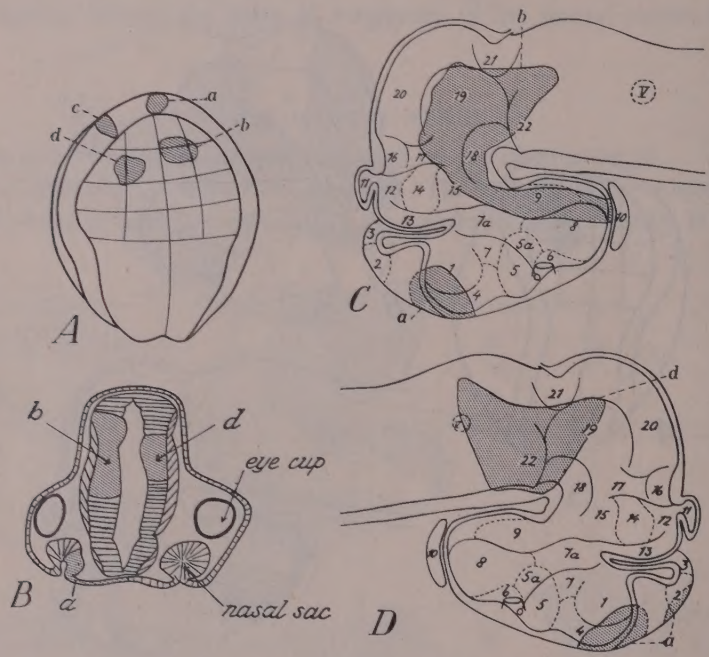
presumptive skin ectoderm. Presumably fixation had been carried out so late that this material had migrated caudally, and had been incorporated there into mesenchymal formations.

The cranial border of the colour mark *b* reaches on the left side the outer margin of the neural plate at a point situated about one-third of the length of zone II behind its anterior margin. In the reconstruction the cranial border of *b* is seen to coincide dorsally with the caudal limit of the cerebellum. Ventrally, and immediately in front of the posterior border of the colour mark, the sulcus isthmi was found. The anterior ventral border lies within the pars ventralis hypothalami. Note that on the right side the stain did not reach anteriorly as far

as the neural ridge. This finds expression in the reconstruction, where the stain does not reach as far up as the dorsal edge, except for a short distance caudally. The point of emergence of the nervus trigeminus was found in the posterior portion of the area stained by *b*. On the left side a dorsal portion of skin had been stained.

Colour mark *c* is situated just caudally to the caudal limit of area IV. In the sectioned larva this mark lay in the foremost part of the spinal cord.

Within the portion of the medulla oblongata, which has been stained by *d*, we find the points of emergence of cranial nerves IX and X. The emergence of the



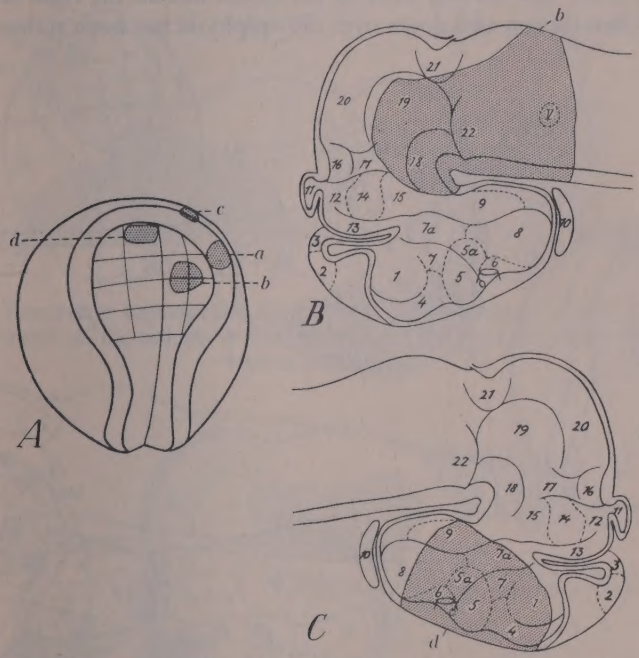
TEXT-FIG. 4. Experimental larva VC 26. *A*: the position of the marks on the neurula. *B*: transverse section through the head of the larva at stage 40, 400 μ from the cranial end. Colour from mark *b* is seen to the right in tegmentum dorsalis and thalamus opticus, from mark *d* to the left in tegmentum dorsalis and from mark *a* ventrally in the skin and in part of the right nasal sac. *C*, *D*: reconstructions of the brain halves at stage 40.

nervus vagus is found just in front of the posterior border of the mark. Nerves VII and VIII emerge 150 and 75 μ respectively in front of *d*. Seventy-five microns behind this mark the medulla oblongata passes into the spinal cord.

VB 121 (Text-fig. 3). Fixed in stage 36. Mark *a* has on either side stained the ventral part of the hypothalamus, the chiasma ridge, the caudal part of the nucleus praeopticus, and part of the optic stalk. In the right eye, in addition, a small part of the retina has been distinctly stained.

The anterior limit of mark *b* coincides with the epiphysis. Part of the skin outside the di- and mesencephalon has been stained.

The fifth cranial nerve emerges just outside and above the posterior part of the portion stained by *c*. Here, as in many other cases, it is apparent that stained cells, which in the medullary plate had been situated distinctly lateral to the median line, are found in the sectioned larva in the floor plate of the neural tube or even some distance across in the opposite wall of the ventricle (VC 35, Text-fig. 6). I cannot so far offer any plausible explanation for this phenomenon.



TEXT-FIG. 5. Experimental larva VC 27. *A*: the position of the marks on the neurula. *B*, *C*: reconstructions of the brain halves at stage 38.

VC 26 (Text-fig. 4). Fixed at stage 40. In addition to the areas indicated in the reconstruction the skin in front of the left hemisphere has also been stained by *a*. Almost half of the nasal sac is also stained (Text-fig. 4B).

b stains the floor of the brain at the level of the dorsal part of the hypothalamus, and also the pedunculus, the dorsal tegmentum, and adjoining parts.

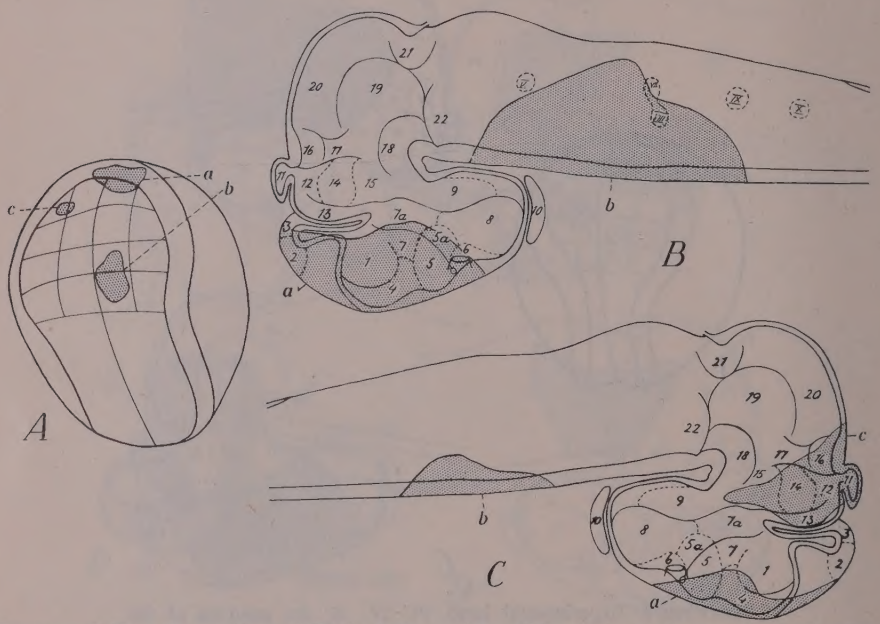
Everything seems to indicate that the part of the neural ridge stained by *c* does not enter into the formation of the brain. The skin, on the other hand, has been stained from a point somewhat behind the epiphysis up to the left ventricle of the fore-brain in the anterior direction.

The emergence of nerve V coincides with the posterior limit of *d*.

VC 27 (Text-fig. 5). Fixed at stage 38. *d*, which marked the anterior half of I B (Text-fig. 1), has stained parts of the area olfactoria and of the septum, the entire nucleus praeopticus, the area strio-amygdaloideus, the chiasma, and parts of the ventral thalamus and hypothalamus. The stain found in the dorsal part of the hypothalamus is puzzling. The left eye-cup is also partly stained.

b has stained the pedunculus, the tegmentum dorsale, the tegmentum isthmi, and an anterior part of the medulla oblongata including the point of emergence of nerve V.

Neither *a* nor *c* has left any trace in the brain; but on the right side the skin above the entire tectum and down over the epiphysis has been stained by *a*.



TEXT-FIG. 6. Experimental larva VC 35. *A*: the position of the marks on the neurula. *B, C*: reconstructions of the brain halves at stage 37.

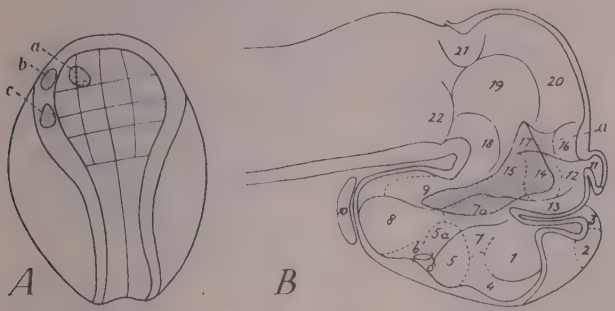
VC 35 (Text-fig. 6). Fixed at stage 37. The part of the neural plate stained by *a* supplies on the right side the anlage of the telencephalon, the anlage of the cranial part of the floor of the diencephalon, and the greater part of the eye-cup; and also the skin, including the nasal placode, external to this region.

Only nerve VIII emerges within the area stained by *b*. Nerve V is situated just in front of and nerve IX behind the colour mark.

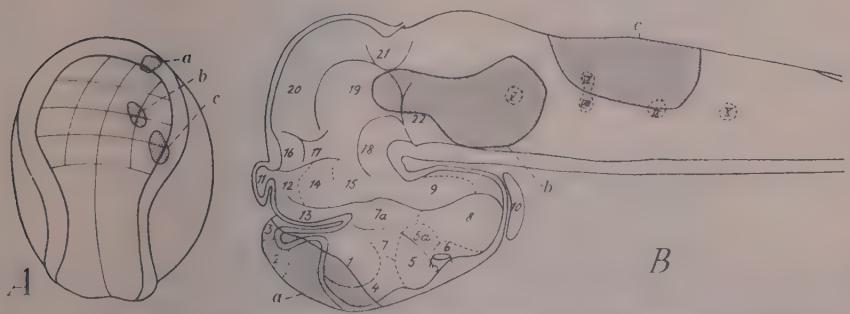
The mark *c* was placed somewhat inside the boundary between the neural plate and the ridge, but in spite of this the stain spreads in the brain right up to the roof of the diencephalon. Probably some diffusion of the stain has occurred here.

VC 38 (Text-fig. 7). Fixed at stage 38. Physiologically the larva had attained the 'early swimming stage' (Coghill). The hemispheres were, however, not quite as developed as they are at this stage according to Herrick.

The stain of mark *a* is situated mainly within the area of the thalamus. There are no traces in the brain of either *b* or *c*. The skin above the diencephalon and the cranial part of the mesencephalon has, on the other hand, been stained by *b*, and the skin upon the crown above the cranial part of the medulla oblongata



TEXT-FIG. 7. Experimental larva VC 38. *A*: the position of the marks on the neurula. *B*: reconstruction of the left half of the brain at stage 38.



TEXT-FIG. 8. Experimental larva VC 44. *A*: the position of the marks on the neurula. *B*: reconstruction of the right half of the brain at stage 38.

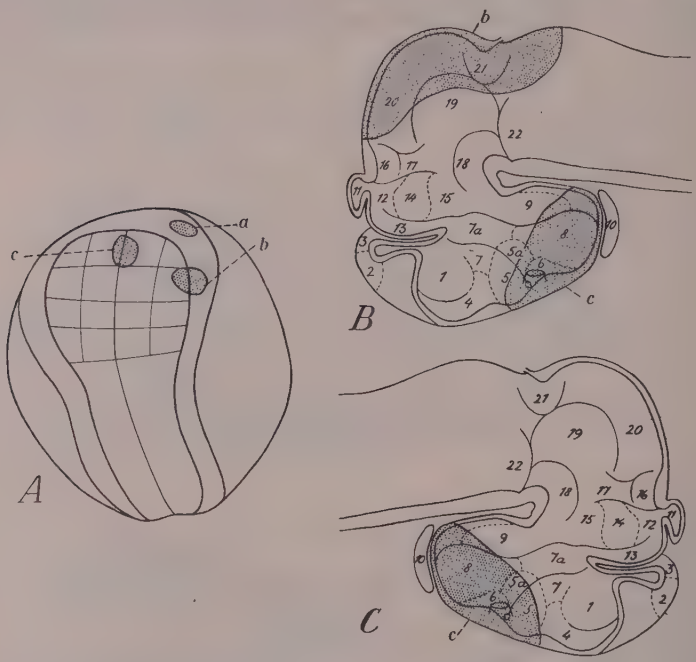
by *c*. The inner part of the neural ridge material coloured by these two marks has presumably been dispersed throughout the body as ectomesenchyme from the neural crest.

VC 44 (Text-fig. 8). Fixed at stage 38. The cranial part of the right hemisphere is stained by *a*. The nasal sac does not seem to be stained, but a small region of skin outside the hemisphere is stained.

The skin above *c* in the medulla oblongata is not stained at all. Of the ganglia, which are at least partly built up by mesenchyme from the neural crest, those of

nerve VII, nerve VIII, and nerve IX exhibit a good blue colour. Because *c* reaches to about half the height of the neural ridge, the inner limit of the skin anlage must run outside it or perhaps upon its very crest.

VC 46 (Text-fig. 9). Fixed at stage 37. Both the skin outside the right hemisphere and the right nasal sac are stained by *a*. *b*, which has stained the tectum



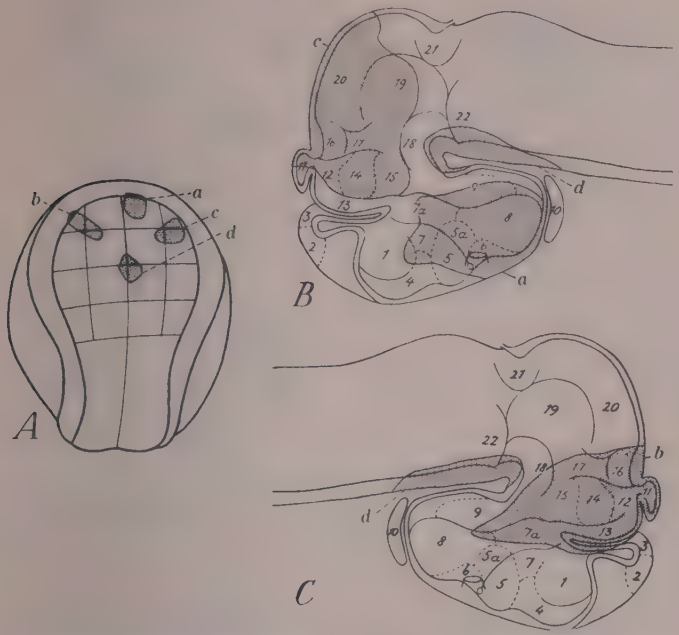
TEXT-FIG. 9. Experimental larva VC 46. *A*: the position of the marks on the neurula. *B*, *C*: reconstructions of the brain halves at stage 37.

opticum, the cerebellum, and a dorsal portion of the foremost part of the medulla oblongata, has coloured also a dorsal portion of skin upon the right side. The anterior edge of *c* coincides with the commissura anterior.

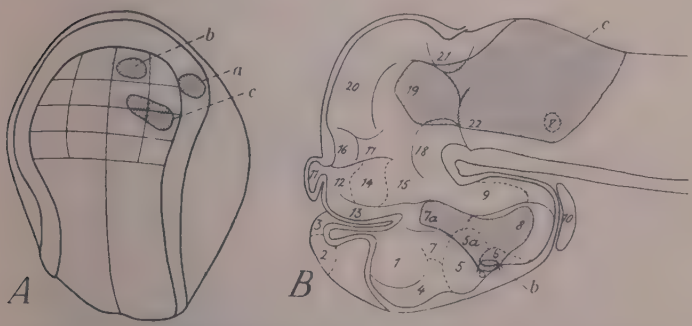
VC 49 (Text-fig. 10). Fixed at stage 38. The ventral hypothalamus, the chiasma, the nucleus praeopticus, the area strio-amygdaloidea, and smaller parts of the area olfactoria and of the area piriformis are stained by *a* as well as the right optic cup with the exception of a minor dorso-cranial area. Upon the left side the dorsal part of the diencephalon plus commissura posterior have been stained by *b*. The mark *c* on the right side is situated caudally to *b*, in agreement with its original position in the plate.

VC 51 (Text-fig. 11). Fixed at stage 38. At the level of the commissura posterior and the tectum opticum the skin of the right side has been stained by *a*. *b* has stained amongst other structures the greater part of the right eye. The

cranial part of the medulla oblongata is stained up to and including the right half of the tela chorioidea posterior in spite of the fact that *c* in the neural plate does not reach the neural ridge anteriorly.

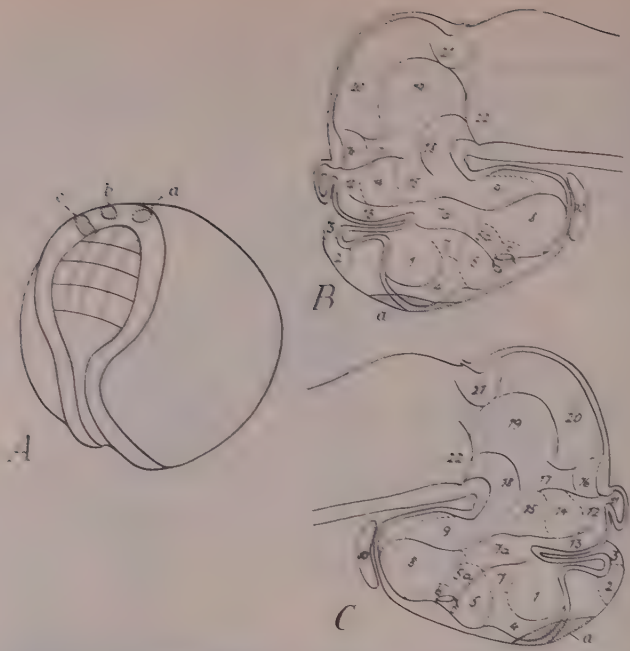


TEXT-FIG. 10. Experimental larva VC 49. *A*: the position of the marks on the neurula. *B*, *C*: reconstructions of the brain halves at stage 38.

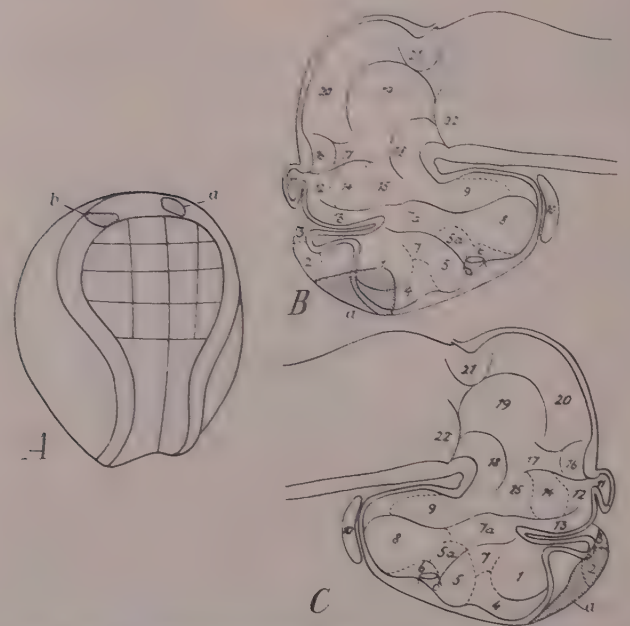


TEXT-FIG. 11. Experimental larva VC 51. *A*: the position of the marks on the neurula. *B*: reconstruction of the right half of the brain at stage 38.

VD 7 (Text-fig. 12). Fixed at stage 39. *b* and *c* have left no trace in the brain. The most ventral part of the primordium of the septum, however, has been stained by *a* which has coloured also a median part of the right nasal sac.

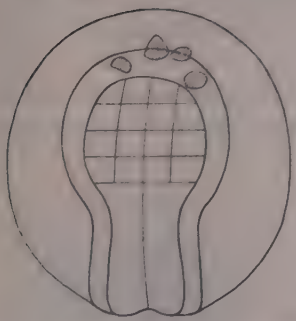


TEXT-FIG. 12. Experimental larva VD 7. *A*: the position of the marks on the neurula. *B*, *C*: reconstructions of the brain halves at stage 39.

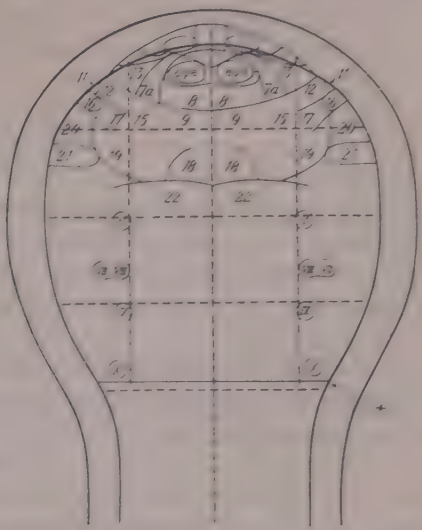


TEXT-FIG. 13. Experimental larva VD 11. *A*: the position of the marks on the neurula. *B*, *C*: reconstructions of the brain halves at stage 37.

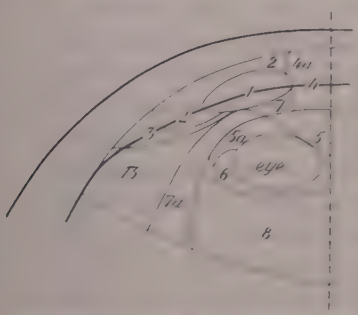
VD 11 (Text-fig. 13). Fixed at stage 37. On the left side *a* has coloured ventral parts of the areae piriformis and hippocampus and also skin including part of the



TEXT-FIG. 14. Experimental larva VD 24. Position of the marks on the neurula.



TEXT-FIG. 15. The presumptive brain areas plotted on to the neural plate (stage 15). For the meaning of the numerals, see pp. 3–4. For the foremost portion, see Text-fig. 16. * = Limit between presumptive brain and presumptive spinal cord.



TEXT-FIG. 16. Detail of the plan of presumptive areas in Text-fig. 15 representing the left side of the anterior part of the neural plate.

nasal sac. Upon the right side the septum primordium and the ventral part of the area olfactoria are stained. The entire right nasal sac is stained. No part of the brain has been stained by *b*.

VD 24 (Text-fig. 14). Fixed at stage 28. None of the four marks which had been placed upon the transverse crest can be discovered within the brain. Parts of the skin at the cranial end of the larva are, however, intensely stained.

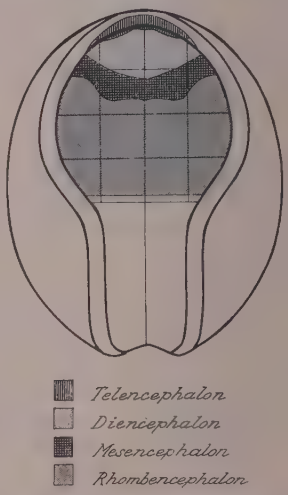
The above experiments, together with 48 similar ones, have formed the basis for the plan of the presumptive areas of Text-figs. 15, 16, and 17.

DISCUSSION

Telencephalon

Text-fig. 17 shows that the presumptive fore-brain of the neurula (stage 15) partly occupies a portion of the transverse ridge and partly the most cranial part of the neural plate (the cranial part of I B, C of Text-fig. 1). Evidence for this distribution is provided by several of the experiments described, e.g. VB 116, VC 26, VC 35, VD 7, and VD 11.

On this question the statements in the older literature by no means agree. Burr (1922) carried out vital staining of the neighbourhood of the anterior neuropore, and also followed the shifting of a hair introduced into it. By doing so he established that the ventral lip of the neuropore is situated just in front of the commissura anterior. The lip itself would then form the lamina terminalis. This opinion is in good agreement with the detailed plan of the presumptive areas (Text-fig. 17). Woerdeman's (1929) investigations with vital staining were aimed at establishing the position of the eye fields, but a study of his published data can, in certain respects, supply information about other presumptive areas. According to his Figs. 9, 11, 14, and 15, the situation of the material for the telencephalon would agree with the results here presented. The colour mark 1, Fig. 9 in his paper, which is applied to the transverse ridge, and which in posterior direction hardly reaches into the pigment groove, is rediscovered in the cranial part of the telencephalon. But in spite of this Woerdeman asserts in the text that the presumptive limit of the neural tube is situated just inside the neural ridge. Manchot's investigation (1929) was on the same line as Woerdeman's. Her Figs. 4 and 6 prove that the anlage of the fore-brain is distributed between the cranial part of the plate and the transverse ridge.



TEXT-FIG. 17. Position of the main presumptive brain regions on the neural plate.

Many authors have based their conclusions about the situation of the presumptive brain areas upon the results of various microsurgical operations. Two types of experiment can be distinguished.

(1) The tendencies towards self-differentiation in different parts of the plate

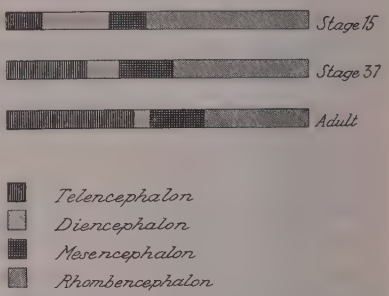
or the ridge have been studied, either after isolations (Mangold, 1933, 1937; v. Aufsess, 1941; Mangold & v. Woellwarth, 1950; v. Woellwarth, 1952) or after implantations in neutral surroundings (Raven, 1935; Alderman, 1935; ter Horst, 1947; Waechter, 1953).

(2) The developed brain has been studied after transplantations within the neural plate or after producing deficiencies in the neural plate (e.g. v. Aufsess, 1941; Hörstadius & Sellman, 1946; Slàdeček, 1952, 1955; Mangold, 1955).

Hörstadius & Sellman (1946) extirpated all the neural plate inside the neural ridge. In spite of this, brain rudiments were formed consisting of two vesicles which were interpreted as the hemispheres of the fore-brain. This led the authors to the conclusion that at least part of the presumptive telencephalon is situated in the transverse ridge. Raunich (1951), who rotated small portions of the neural plate, arrived at the same conclusion. The transplantations carried out by Alderman in 1935 also yielded results which seem to agree with those of the present author. Material from the foremost part of the neural plate inside the neural ridge was transplanted to the ventral side. In all cases a differentiation towards diencephalic tissue was observed, and in some cases also parts of the telencephalon were formed. The fact that the latter did not always occur might be explained by the difficulty of placing the cut exactly at the boundary between plate and ridge. This experiment can, however, hardly be said to prove that part of the presumptive telencephalon is situated in the neural plate proper. The cut might have been slightly misplaced so as to include a little of the transverse ridge; or the occurrence of telencephalic formations might be due to regulation.

I am at all events inclined to explain the discrepancies between my own results and those obtained by the authors now to be discussed by the great regulatory ability of the material of the neural ridge. According to these authors the transverse ridge contains not only material for the telencephalon, but also the entire presumptive prosencephalon. This view is maintained by Slàdeček (1955), who rotated one of the lateral halves of the cranial part of the neural plate through 180°, with the result that the prosencephalon developed normally in the anterior part of the head, while the remaining parts of the brain on the operated side are arranged in inverse order. The operation thus resembles that carried out by Hörstadius & Sellman (1946) and by Raunich (1951). Another explanation of the discrepancy might be supplied by the fact that many of Slàdeček's operations were carried out when the larva had already reached stage 16. According to Baker & Graves (1939) part of the foremost portion of the plate is depressed below the transverse ridge during the later stages of the neurula. At least the cranial part of the presumptive diencephalon, which according to my results lies within the anterior third of area I in stage 15 (Text-fig. 15), ought by stage 16 to have had time to sink below the transverse ridge. This reasoning is in agreement with many of my experiments in which a colour mark placed some distance caudally to the transverse ridge in stage 15 had in stage 16 moved forward into the immediate neighbourhood of the ridge. Waechter (1953) in her plan of

differentiation ability of the different areas (Fig. 16 in her paper) has also placed both telencephalon and diencephalon in the transverse ridge. She obtained her results by inserting into the blastocoele of a young gastrula different parts of the neural plate or the neural ridge, and by examining later the differentiation of the graft. Although Waechter does not directly use the term 'plan of anlages' when referring to her figure, she says nevertheless (p. 250): 'Die Implantate differenzierten sich im wesentlichen herkunftsgemäß, wobei die Bereiche der "potentiellen Anlagen" die der "materiellen Anlagen" nur wenig überschritten.' Such a statement requires a knowledge of the pattern of anlagen in the neural plate, and this she derived from the results of earlier investigators who worked with transplantation and isolation (Mangold, 1933; Raven, 1935; v. Aufsess, 1941; and others). As a matter of fact some reservations have already been made (e.g. by Raven, 1935) concerning these earlier results with regard to possible erroneous conclusions due to the disregard of regulation. It is significant that Waechter is doubtful of the validity of her results only with regard to one presumptive organ, and there considers the possibility of regulation, and this is in the case of the eye-forming area, the position of which had already been investigated by means of vital staining.



TEXT-FIG. 18. Relative size of the main brain regions at different stages of development.

This investigation has shown that the presumptive fore-brain occupies only a relatively small portion of the brain-forming area. Text-fig. 18 illustrates the relative size of the major brain regions at different stages of development. From this figure it is evident that the telencephalon has, beyond comparison, the greatest relative growth.

Diencephalon

The experimental larvae earlier described, VB 121, VC 35, VC 46, VC 49, and VC 51, supply information about the position of the presumptive diencephalon (Text-fig. 16). All material of this brain region is found in the plate proper.

If I have interpreted correctly Woerdeman's figures (1929), his results with vital staining support my conception. In Woerdeman's Fig. 9 the posterior part of colour mark 2 seems in the reconstruction to be found in the region of the recessus opticus, mark 3 in the hypothalamus, mark 4 in the cranial part of the region of the thalamus, the region of the habenula and of the epiphysis. In his Fig. 11 the posterior and greater part of mark 2 and the anterior part of mark 3 would be discovered in the diencephalon. In his Fig. 14 the lateral part of mark 2 seems to form the roof of the diencephalon, and in Fig. 15 the region around the epiphysis and the ganglion habenulae appears to have originated from material

stained by mark 4. To agree with my results marks 2 and 3 ought, however, to have stained the major part of the telencephalon. According to his Fig. 15*e* they seem to have been rediscovered almost exclusively within the diencephalon.

Lehmann (1945) has suggested that the prechordal mesoderm forms a specific inductor of prosencephalic tissues. According to Nieuwkoop (1952, Fig. 2B) the prechordal region of the neural plate coincides with the part which according to the present investigation forms the presumptive prosencephalon.

Mesencephalon

Information about the localization in the neural plate of the anlage of the mesencephalon is provided by, for example, larva VB 121. Larvae VC 27 (Text-fig. 5), VC 38 (Text-fig. 7), VC 51 (Text-fig. 11), and VD 7 (Text-fig. 12) supply strong evidence against the participation of the neural ridge in the formation of the mid-brain. In all these cases the neural ridge has been stained at the level of the presumptive mesencephalon right to the border of the plate proper. In none of these cases has brain tissue been stained. Thus I cannot agree with Mangold (1937) who, on the basis of transplantations and isolations, asserts that the ridge participates in the formation of both mesencephalon and rhombencephalon.

Rhombencephalon

A great number of experiments, e.g. VC 44, VC 49, and VC 51, supplied information about the position in the neural plate of the presumptive hind-brain.

It is of particular interest to be able to determine the posterior limit of the presumptive medulla oblongata. According to Manchot (1929) the presumptive brain forms two-thirds of the neural plate at the time of the latter's first appearance, while after the formation of the neural ridge it occupies half the plate. This agrees excellently with the results presented here. The zones I–IV are intended to occupy just half of the neural plate, and according to the experiments (e.g. VB 116) the presumptive spinal cord begins in the caudal part of zone IV. The results of transplantations and isolations point, however, in entirely different directions. According to Waechter (1953) regions which correspond to at least the entire zone IV exhibit ability to self-differentiate towards spinal cord, while ter Horst (1947) found as far back as the 4th (from the anterior) fifth of the plate, an ability to differentiate more strongly towards medulla oblongata than towards spinal cord.

The eyes

Many experimental embryologists have investigated the position in the neural plate of the eye-forming area. Woerdeman (1929) and Mangold (1931) supply extensive reviews of the older literature. Both Woerdeman (1929) and Manchot (1929) have attempted to answer the question with the aid of vital staining, and have concluded that in a neurula, corresponding nearly to stage 15, the anlage

is situated in the plate in a far anterior and lateral position. This conception has been criticized by Hörstadius & Sellman (1946) who believe that in this case the recessus opticus would occupy a dorsal position after the neural tube had been closed.

In two experiments (VB 129 and VC 25) the colour marks were placed just inside the ridge in the region where, according to Woerdeman, the anlage of the eyes should reach the extreme edge of the plate. In none of these experiments have the eyes been stained. If, on the other hand, the position of the eye-forming area is that deduced from the present experiments (Text-fig. 15), it becomes much easier to understand how the later topography of the opticus region comes into existence.

The external limit of the brain-forming area

The limit between presumptive brain and presumptive skin has previously been dealt with in a number of papers based upon vital staining. Woerdeman (1929) and Fautrez (1942) were of the opinion that the boundary coincides with the outer limit of the neural plate. Already in 1939 it had, however, been clearly shown by Baker & Graves that the inner half of the transverse ridge takes part in the formation of the brain. The same result was reached by Hörstadius & Sellman (1946) who, on the basis of their own results and those of earlier authors, discussed the problem in detail. Their result differs from those of the present investigation in that they indicate (in their Fig. 5c, p. 15) that material belonging to the first four of the zones distinguished by them in the neural ridge participates in the formation of the brain. According to my division these zones would closely correspond to the ridge level with I and half of II (Text-fig. 1). Experiments VC 26, VC 27, VC 38, VC 51, VD 7, and VD 11 show that the limit of the presumptive brain cannot be situated upon the ridge farther back than the region which corresponds to Hörstadius & Sellman's zone 1 plus the cranial part of zone 2. Experiment VD 24 and others show that this limit, where it is situated upon the transverse ridge, must be drawn as in Text-fig. 16.

The neural crest material, which is later dispersed throughout the body as ectomesenchyme, is situated between the presumptive brain and the presumptive skin. However, the larvae stained in the ridge proper are at present too few to permit a safe mapping of this material.

The nasal and auditory placodes

With regard to the localization of the anlagen of these placodes the author has arrived at the same conclusions as those of Carpenter (1937) and Röhlich (1929) in their experiments with vital staining. Thus the anlage of the nasal placodes was found on the outer part of the transverse ridge and on the epidermis outside it, roughly on a level with the middle of zones I B and I C (Text-fig. 1). The presumptive auditory placodes are situated just outside the ridge on a level with zone III.

The ability to regulate at the neurula stage

A number of authors, who have studied the ability of the neural crest and the neural plate to self-differentiate, have considered the degree of determination in the developing brain at the neurula stage. Waechter (1953) thinks it probable that the material of the neural plate is almost finally determined. Raven (1935) did not feel able to decide the degree of determination on the basis of results obtained by transplantations. ter Horst (1947), on the other hand, obtained in the course of her experiments results which afforded evidence for the occurrence of strong regulation.

A review of the tendencies towards self-differentiation in the light of the results of the present investigation shows the prospective potency in the different regions of the neural plate to be much wider than their prospective significance. Roach (1945) has shown the cranio-caudal polarity to be determined at stage 13, and Slàdeček (1952) has demonstrated that the mediolateral polarity is settled in stage 15. It is probably, however, still a far cry from here to the final determination of all cells. We have to count with the possibility that the different potencies still form an overlapping system, so that upon isolation one region is able regulatively to realize also the prospective significance of the regions next to it. The particularly strong regulative ability of the neural ridge is remarkable. This is excellently illustrated by Mangold's experiments (1937) in which he isolated a piece of the ridge roughly on a level with my zone III (Text-fig. 1). This isolated portion differentiated diencephalic, mesencephalic, and rhombencephalic tissue, and also eyes and nasal sacs.

SUMMARY

1. By means of vital staining it has been possible to project the most important areas of the fully developed brain of the axolotl, *Siredon mexicanum*, upon the neural plate of the larva. The map of presumptive areas is shown in Text-figs. 15–17.

2. The investigation has shown that the presumptive fore-brain occupies space not only upon the foremost part of the neural plate, but also upon the transverse ridge.

3. All material of the presumptive diencephalon and of the more caudally situated brain regions is situated in the plate proper.

4. The position of the eye-forming area has been shown to be more medial than has been proposed by earlier authors.

5. This mapping of the presumptive brain areas makes it possible to judge with greater certainty the question of self-differentiation and regulation in the transplantation experiments of previous authors.

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Studies in Embryonic and Larval Development in Amphibia

I. The Embryology of *Eleutherodactylus ricordii*, with Special Reference to the Spinal Cord

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WITH ONE PLATE

INTRODUCTION

AMONG the Amphibia there are numerous examples of the suppression to a varying extent of a larval stage in the life-history. In such instances the animal is freed by various means from the necessity of passing its early phases of development in open water. This evolutionary trend has nowhere proceeded further than in the Anuran genus *Eleutherodactylus*, which is distributed through the Caribbean and the adjacent mainlands. In *Eleutherodactylus*, development is direct and wholly embryonic, and many larval features have been suppressed.

In 1871 was published the first description of a West Indian frog which laid eggs in air, and from which young frogs with fully formed limbs were hatched (Bello y Espinosa, 1871). Since that time some twenty papers have been published on the embryology of different species of the genus, mainly in recent years by Dr. W. Gardner Lynn and his collaborators. The main account of the general development of *Eleutherodactylus* relates to the montane species *E. nubicola* (Lynn, 1942). Later papers have been concerned with the role of the thyroid gland in this Amphibian which develops without any metamorphosis (Lynn, 1948; Lynn & Peadon, 1955; Millott & Lynn, 1954).

In *Eleutherodactylus* the eggs are large and yolky and are enveloped in layers of tough jelly. The segmentation is complete, though for much of embryonic life the bulk of the embryo consists of a mass of large yolky cells surmounted by a cap formed by the smaller-celled dorsal region of the embryo. Waddington (1952) has remarked on the tendency towards the development of a blastoderm among the more yolky eggs of Amphibia. In this direction *Eleutherodactylus* approaches the limit consistent with holoblastic cleavage.

In the embryo of *Eleutherodactylus* the gill clefts are never open, and the only

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complete aortic arches ever to be formed are the carotid and systemic. There are no lateral-line organs or nerves; no tadpole-like mouth parts or coiled gut. In some species there are traces of external gills.

The present study is mainly concerned with some aspects of neural development in *Eleutherodactylus*. The nervous system of the developing Amphibian shows a number of primitive fish-like features which have persisted as larval adaptations. It is consequently of interest to examine how far they remain in an Amphibian with an embryonic type of development. One instance of the atrophy in *Eleutherodactylus* of such a feature has already been observed by Dr. Lynn. Not only have the lateral-line organs been lost, but also the two giant Mauthner neurones of the medulla are absent (Lynn, 1957). In fishes and larval Amphibia, these cells form the middle link in a three-neurone chain between the vestibular system and the trunk musculature. In most Anura the Mauthner cells disappear at metamorphosis, though they persist in the aquatic genus *Xenopus* (Stefanelli, 1949).

In the present paper are also described observations on the development of the dorsal root ganglia and ventral motor horns of *Eleutherodactylus*, the interest of which are neuro-embryological rather than phylogenetic. It is intended, however, in a second paper to return to the theme of larval and embryonic development by a comparison of the motor innervation of trunk and limbs in *Xenopus* and *Eleutherodactylus*.

MATERIAL

Eggs of two species of *Eleutherodactylus* were collected in the neighbourhood of the University College of the West Indies, Mona, St. Andrew, Jamaica. Two clutches each of about a score of eggs were found by Mrs. Valerie Croston, in her garden at College Common; they had been laid in coco-nut husks in which orchids were growing. With the second batch was a female of *E. ricordii*. These eggs were placed on moist filter paper in Petri dishes and were observed in the laboratory from day to day. Generally, three eggs from each clutch were fixed on each day. The first batch was obtained 5 days before hatching, and the second 11 days. The development of both hatches during the last 5 days of intra-oval life appeared to be identical. They have both been assigned to *E. ricordii*. Goin (1947) states that in Florida the average period of development of this species is 15·6 days.

In addition, one clutch of eggs was collected from the situation where Dr. Lynn has found that those of *E. martinicensis* are laid (Lynn, 1957), namely, in the axils of the leaves of the wild 'pinguin', a Bromeliad, 'vigorous and formidable', as Gosse (1851) describes it, 'the recurved spines with which the edges of the long leaves are set being exceedingly sharp, and inflicting terrible scratches'. This third batch of eggs was collected at a stage which corresponded in most respects with that of *E. ricordii* 5 to 6 days before hatching. These eggs of *E. martinicensis* were in process of hatching after 6 days in the laboratory. They were clearly

different from those of *E. ricordii* in that the embryos were more heavily pigmented and somewhat larger in size though the diameter of the outside of the egg was the same in both species, namely, 3·5 mm. According to Lynn & Peadon (1955) the normal developmental period of *E. martinicensis* is 13–14 days. As in *E. ricordii*, embryonic life ends with the greater part of the yolk yet to be absorbed.

METHODS

Each embryo was dissected free of its jelly-layers and fixed for 24 hours in a 1 : 1 mixture of Bouin's fluid and cellosolve, as recommended by Lynn (1948). The material was then gradually dehydrated in ascending mixtures of cellosolve and water. From pure cellosolve the embryos were transferred to a 1 : 1 mixture of xylene and cellosolve, and then cleared in pure xylene. They were then embedded in paraffin wax of m.p. 60° C. Sections were cut at 9 μ. Some series were stained in Ehrlich's haematoxylin and eosin while others were impregnated with silver by Bodian's method, for which the silver proteinate of Établissements Roques of Paris was used.

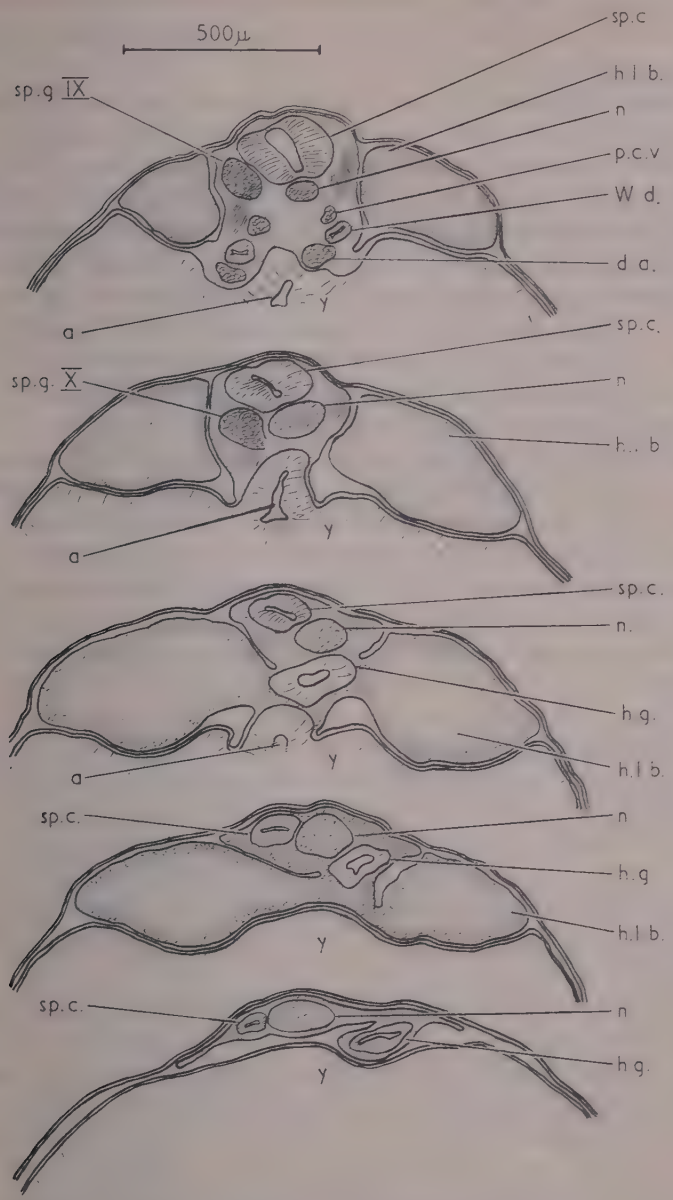
Serial sections were also prepared from the central nervous system of the adult *E. ricordii*. The animal was killed in Bouin's fluid and, after death, the dorsal surface was skinned and an axial strip of the body was cut out, some 5–6 mm. wide and less than 20 mm. in length. This contains the whole of the central nervous system. Fixation was continued for a further 24 hours, after which the specimen was decalcified in 1 per cent. nitric acid in 70 per cent. alcohol, dehydrated in ascending alcohols, and then cleared in xylene and embedded in paraffin wax. Sections were again cut at 9 μ and stained by the same methods.

GENERAL DESCRIPTION OF DEVELOPMENT IN *E. RICORDII*

The second batch of eggs were collected on the 15th of January 1958, and those which remained unfixed, hatched on the 27th of the month. Following the usage of Lynn (1942), the stages of development of the material will be designated by the number of days which remained before hatching.

–11 days. The embryos were at a stage with small limb-buds. Most of them were rotating within the egg-envelopes in the way described by Gitlin (1944) for *E. portoricensis*. Of 14 embryos in movement, 2 were rotating clockwise and 12 anti-clockwise.

In section series deep clefts are seen in the surface of the embryo between the limb-buds and the upper part of the body-wall. The tail-bud is present. Eye-vesicles have appeared, and the hind-brain is thin-roofed. In the auditory sacs the endolymphatic duct is yet undilated. The fore-gut is a flattened diverticulum of the archenteron, with which it joins by a wide anterior intestinal portal. The heart is a simple tube with endo- and myocardium. The three pronephric tubules



TEXT-FIG. 1. Drawings from section series through embryo of *E. ricordii* at −10 days, to show transition from trunk region (top of page) to tail, flattened on one side against egg-membrane. *a*, archenteron; *d.a.*, dorsal aorta; *h.g.*, hind-gut; *h.l.b.*, hind limb-bud; *n.*, notochord; *p.c.v.*, posterior cardinal vein; *sp. c.*, spinal cord; *sp. g. IX*, *X*, spinal ganglion IX, X respectively; *W.d.*, Wolffian duct; *y*, yolky endoderm.

have open funnels into the coelom. The Wolffian duct extends backwards to the level of the hind limb-buds. The myotomes consist of elongated myoblasts, which as yet show no cross-striations.

–10 days. The limb-buds were larger. The embryos were no longer rotating, but muscular movement had begun; in sections cross-striations were evident in the trunk myotomes. Both spontaneously and after slight mechanical shock, the embryos would wriggle in a way corresponding to the 'early flexure' stage of activity in *Ambystoma* (Coghill, 1929) (in later stages this motion was never seen to progress any farther towards swimming movements). By –10 days the nasal placodes have appeared, and the eye-cup is two-layered; it contains a hollow lens vesicle. A large infundibular recess has appeared on the floor of the mid-brain. On the surface of the third pharyngeal arch at this stage is a blunt vascular papilla which may represent an external gill, though considerably smaller than Gitlin (1944) has described in *E. portoricensis*.

The stomodaeum is present, though it does not yet make contact with the pharynx. The anterior intestinal portal is much narrower; on its anterior surface are seen the first epithelial cords of the liver. Posteriorly, the archenteron leads into the hind-gut. The latter is continued by an open proctodeal tube. The tail, still closely applied to the surface of the egg, is twisted through a right angle, so that its neural tube and notochord lie side by side (Text-fig. 1). A deep cleft leads from the medial surface of one hind limb-bud beneath the lateral surface of the apposed tail.

–9 days. By this stage new features were apparent in the living embryos. The heart was beating and blood was in circulation. The whole egg could easily have been mistaken for that of an Amniote, with a network of blood-vessels over the yolk, and the vascular tail, now closely applied to the inner surface of the egg envelope, strikingly resembling an allantois. The eyes were pigmented.

The olfactory placodes have become cup-shaped. The inner layer of the lens vesicle is thickened. The saccus endolymphaticus, now separated from the original auditory vesicle, is beginning to expand over the hind-brain as in other Amphibia (Whiteside, 1922).

–8 days. Over the whole body surface melanophores have developed pigment. The limbs now protrude stiffly from the body and are several times as long as broad. The alimentary tract consists of two narrow tubes, suspended by mesenteries, which lead respectively from pharynx and cloaca to separate junctions with the central mass of cellular yolk—as yet little reduced in volume. The lumen of the fore-gut tube in the oesophageal region is occluded. Oesophagus and trachea have become separate, and the latter ends in the dilated lung-buds.

–7 days. The hind limb is now rather longer than the forelimb, and ends in a spatulate tarsal region. The internal nares now open into the pharynx. In the eye the lens fibres are heavily cornified. The inner ear has acquired two semicircular canals, and the future position of the third is indicated. Within the heart the truncus arteriosus is divided by a septum, and the trabeculae of the

ventricular wall are developing. The auricle is yet undivided. The first mesonephric tubules have appeared.

−6 days. The first signs of layering within the retina are apparent. The auricular septum is present, and in the ventricle the trabeculae are much deeper. Within the thyroid, vesicles have developed.

−5 days. The egg-tooth at the tip of the upper jaw has appeared. The digits are visible in both pairs of limbs. A second fibre-layer can be traced within the retina. Chondrification has begun at several points within the skeleton; the hind limbs and pelvic girdle are more advanced in this respect than are the anterior members. Cartilage matrix has appeared within the first few vertebrae. No degenerative changes within the pronephros are yet seen.

−3 days. In both humerus and femur the cartilage of the shaft is hypertrophic, and invasion by osteocytes and blood-vessels has just begun. Two membrane bones are present in the head; the squamosal lateral to the auditory capsule, and the dentary along the inner surface of Meckel's cartilage. The mesonephros is much larger than at −5 days, and within the pronephric tubules vacuolation is apparent. The mucous glands within the skin now have acini.

−1 day. The first signs of ossification are visible in the distal limb segments. Within much of the chondrocranium cells have become hypertrophic. To external view the tail is yet undiminished, yet the first degenerative change is apparent in sections, in that the ectodermal cells of the tail have put out irregular amoeboid projections. The pronephros is now a mass of vacuoles.

Comparison of the rate of development of *E. ricordii* with *E. nubicola* (Lynn, 1942) with respect to the features described above indicates that −11 days in *ricordii* corresponds to −20 days in *nubicola*, and that the former hatches at a stage which in many respects resembles that of the *nubicola* embryo with 7 days of the intra-oval period yet remaining.

Free life in *E. ricordii* begins with the greater part of the abdominal cavity still occupied with yolky cells unchanged since the period of cleavage, and with the later stages of yolk absorption, such as Sampson (1904) and Lynn (1942) describe, still to be accomplished. In both *E. ricordii* and *E. martinicensis* the rate of development must be among the most rapid of all vertebrate ontogenies. Three days after the lens vesicle has invaginated its inner wall has formed heavily cornified lens fibres; the limb-skeleton begins to ossify 2 days after the first appearance of cartilage matrix. The same rapidity of development is seen within the central nervous system.

THE DEVELOPMENT OF THE SPINAL CORD IN *E. RICORDII*

At −11 days the spinal cord, as yet confined to the trunk region, consists of a thick wall on each side, joined by thin roof and floor plates (Plate, fig. A). The wall is about 80 μ in thickness at most. The nuclei are about 10 μ long, and occur in every position throughout the depth of the epithelium. Mitotic figures are

common near the ependymal surface; yolk granules are still scattered throughout the neural tube. This trunk cord of *Eleutherodactylus* differs in several ways from that of other developing Amphibia, but resembles the cord of other vertebrates with embryonic development, such as the dogfish or the chick. In an early Amphibian larva the inner cellular part of the cord consists only of a single inner ependymal layer, surrounded by a zone of functional neurones which soon are clearly differentiated from other cells; mitotic figures soon become infrequent among the ependymal cells. The embryonic type of cord, on the other hand, laterally consists of a thick neuroepithelium through which the nuclei migrate to divide at the ependymal surface, from which they afterwards retreat (Sauer, 1934). In *Eleutherodactylus* mitosis within the trunk cord does not cease until 5 days before hatching.

The tail cord of the *Eleutherodactylus* embryo is similar to that of other young Amphibia in calibre. It is first recognizable in *E. ricordii* at −10 days. The cord then sharply tapers at the junction between the two zones, where it twists through a right angle to run alongside the notochord of the tail.

At the surface of the trunk cord at −10 days are first seen a thin layer of longitudinal fibres which are part of a descending tract which originates in the medulla oblongata. Within the myotomes fine single nerve fibres are seen in the plane of the myocommata. At these sites in other Amphibia both the first sensory and motor fibres are found (Hughes, unpublished), though to which category in *Eleutherodactylus* these first myocommatal fibres belong, I am unable to decide.

The dorsal root ganglia, which on the previous day were part of a continuous tract of neural crest cells, are now, at −10 days, separately discernible. Within the anterior trunk ganglia some of the constituent neuroblasts have produced a short, outwardly directed primary nerve fibre.

At −9 days another set of sensory cells has appeared; these are the Rohon–Beard neurones, which are found in all lower vertebrates. They remain within the cord and form a primary and transitory sensory system. *Eleutherodactylus* is so far unique in that their appearance follows that of the dorsal root ganglia. At −9 days in *E. ricordii* they are found in dorso-lateral positions within the cord, in both trunk and tail (Plate, fig. D). They are pear-shaped, with a large nucleus, which differs from that of the surrounding neuroblasts in that there is a clear nuclear sap containing a large nucleolus. The apex of the cell is continued by a single nerve process.

A further feature at this stage is the outgrowth of the definitive spinal motor roots. At trunk levels bundles of extremely fine fibrils emerge from the ventral surface of the cord, opposite the dorsal root ganglia, beneath which, and accompanied by numerous future Schwann cells, they make their way to the myotomes. Each bundle of motor fibres then turns caudally to run along the inner surface of a myotome as far as the adjacent inter-myotomic boundary into which a few motor fibres can now be clearly traced. A primary myocommatal innervation of the somatic musculature has already been demonstrated in the larva of *Xenopus*

(Lewis & Hughes, 1957) where a histochemical reaction for cholinesterase is first detectable at these sites (Lewis, 1958).

By −9 days in *E. ricordii* a bundle of fine nerve fibres has appeared in each limb-bud (Plate, fig. J). Some of these fibres can be traced back through motor roots to ventral horn cells at limb levels in the cord. The ventral horns at this stage form an expanded lower zone of the mantle layer of the cord in which many cells have already developed into bipolar neuroblasts with a slender nerve process at each end (Plate, fig. E). Their axons point in a ventro-medial direction. These ventral horn cells are further distinguished from neuroblasts elsewhere in the mantle layer by their larger, clearer nuclei; each has a single large nucleolus. Elsewhere in the trunk cord motor neuroblasts are not conspicuous, though in the tail pairs of primary motor neurones are recognizable. Within the dorsal root ganglia differentiation of neuroblasts is proceeding, and occasional cell-bodies are conspicuous by the size of their nuclei. At −8 days fibres from the dorsal root ganglia first penetrate the cord to enter the dorsal funiculus, within which dorsal root fibres bifurcate and give off branches which run longitudinally in both directions. At this stage the general form of the trunk cord in *Eleutherodactylus* is still further unlike that of corresponding stages in a larval Amphibian, where the white matter of the cord forms a broad even margin to the inner cellular layers. In *Eleutherodactylus*, however, the fibre tracts are much sparser, and dorsally do not reach beyond the dorso-lateral sensory funiculus. This feature is associated with the early development of the dorsal root ganglia relative to that of the Rohon–Beard system of neurones. At −7 days in *Eleutherodactylus* the trunk cord is strikingly reminiscent of that of a 6-day chick embryo (Plate, fig. C).

Not until −8 days in *E. ricordii* can fibres which belong to the Rohon–Beard cells be traced. From each Rohon–Beard cell two processes are given off; one continues as an afferent fibre which leaves the dorsal surface of the cord; from the second, a longitudinal fibre joins the dorsal funiculus (Plate, fig. I). Both processes may arise from a single short axon.

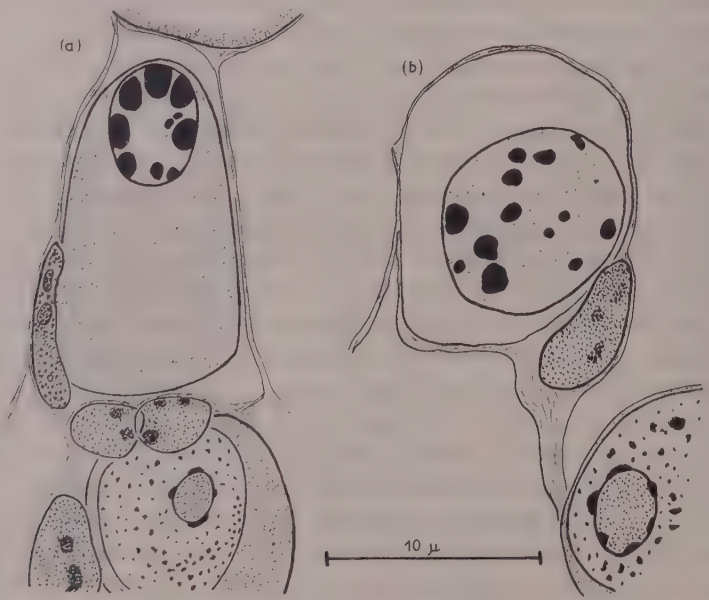
From −7 days onwards Rohon–Beard cells migrate towards each other to meet in the mid-dorsal plane. This movement is part of a general inward folding of the dorsal part of the spinal cord which thereby greatly increases in thickness. For *Xenopus* this movement has already been described (Hughes, 1957). In *Eleutherodactylus ricordii* the shift in position of the Rohon–Beard cells is achieved in the tail at −7 days, but is not completed in the thicker cord of the trunk region until 2 days later (Plate, figs. G, H). Towards the end of development Rohon–Beard cells become fewer in number, but in *E. ricordii* are still numerous at the root of the tail on the day before hatching.

Meanwhile, differentiation continues within the ventral horns of the cord. At −9 days these centres are first recognizable as ventro-lateral expansions of the mantle layer (Plate, fig. B). As development proceeds they become increasingly distinct from the adjacent masses of neuroblasts. By −6 days the main part of the ventral horn consists of a compact ovoidal mass of bipolar cells whose

axons converge towards the ventral surface of the cord. Below them is a smaller subsidiary group of neuroblasts. The ventral horn is now similar to that of late larval stages in *Xenopus*, whereas in its first appearance there was a resemblance in general form to that of a 3-day chick embryo. At −1 day the ventral horn cells have reached a condition of considerable maturity. Blocks of Nissl material are visible in the cytoplasm towards either pole of the cell. The linear dimensions of the nucleus are about twice that in neighbouring cells.

CELL POPULATIONS IN VENTRAL HORNS AND DORSAL ROOT GANGLIA

From −7 days onwards in *E. ricordii* pycnotic nuclei are found among the ventral horn cells. Here cell degeneration is at a maximum from −5 to −3 days, and becomes infrequent on the day before hatching. The loss of some maturing ventral horn cells in developing Amphibia has also been observed in *Xenopus* (Hughes & Tschumi, 1958). In *Eleutherodactylus*, however, pycnotic nuclei are especially frequent at these sites.



TEXT-FIG. 2. Stages of cell degeneration in differentiated neurones, from the second dorsal root ganglion of an embryo of *E. ricordii* at −5 days. Taken from contiguous sections. (*a*), nuclear membrane still present, and cytoplasm as yet unchanged. Chromatin within nucleus aggregated into lumps; (*b*) shrunken remains of cell enclosed by original capsule. Satellite cell still present.

In the dorsal root ganglia also degenerating cells are common during the same period of development. At −7 days pycnotic nuclei and mitotic figures occur together among neuroblasts which as yet show little differentiation. At later

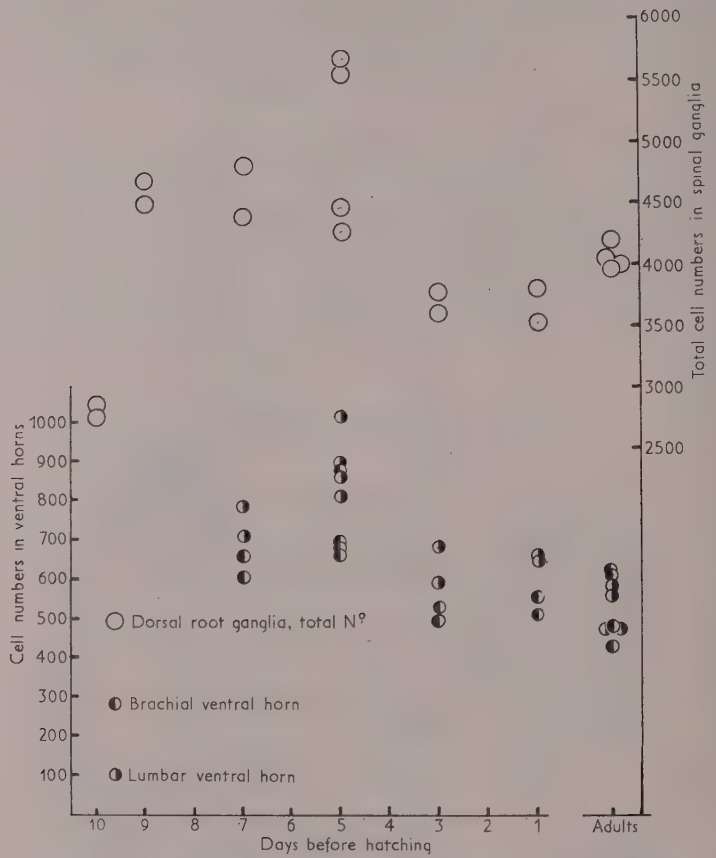
TABLE 1

Counts of cells in dorsal root ganglia of E. ricordii. *Two individuals were counted at −5 days, and also in the adult stage*

Dorsal root ganglia	*Days before hatching*														*Adult individuals*			
	10		9		7		5		5		3		1					
1	155	244	151	236	316	430	175	186	223	281	138	108	70	112	168	191	133	195
2	619	629	804	959	1,066	1,011	970	973	1,357	1,504	840	988	781	951	659	727	914	954
3	268	141	369	456	391	335	352	369	466	437	184	229	238	216	275	224	404	524
4	202	129	254	299	273	212	248	257	243	226	218	203	142	204	239	275	268	323
5	157	150	206	245	220	209	213	192	248	312	185	193	126	195	293	239	219	228
6	166	247	245	221	215	197	252	217	270	278	132	135	132	153	187	201	149	195
7	254	227	369	441	405	363	388	333	411	432	256	281	281	316	320	345	142	231
8	320	364	768	555	692	540	598	611	732	656	562	536	582	585	586	551	584	623
9	448	447	1,005	857	733	584	813	820	1,059	963	637	701	752	713	765	783	752	563*
10	268	138	296	388	464	466	431	380	648	430	435	395	406	349	509	494	420	376
TOTAL	2,829	2,716	4,467	4,657	4,775	4,347	4,440	4,238	5,657	5,519	3,587	3,769	3,510	3,794	4,001	4,030	3,985	4,212

* Some sections missing.

stages of embryonic life the various phases of cell degeneration (Glücksmann, 1930) are well shown among the larger neurones of the dorsal root ganglia (Text-fig. 2). The frequency of pycnotic nuclei in the spinal ganglia and ventral horns of *Eleutherodactylus* suggested an inquiry into the effect of this loss of cells



TEXT-FIG. 3. Average totals of cells in spinal ganglia in *E. ricordii* at the stages indicated, together with cell numbers in ventral motor horns.

on their total numbers within these sites throughout development. These counts have been continued into the adult stage of the life-history.

Compared with other Anura the adult of *Eleutherodactylus* is very small. The central nervous system has been adapted to these reduced dimensions apparently in two ways—either by decrease in cell size or by reduction in total numbers as with the motor neurones within the cord and the cells of the dorsal root ganglia. Thus whereas in *Xenopus* at metamorphosis the larger spinal ganglia may contain about 10,000 cells, the corresponding number in an adult *Eleutherodactylus* is about a twentieth of this figure. The largest mature neurones of the dorsal root

ganglia are about 40 μ in diameter and hundreds of times the volume of the majority of cells within the cord.

The results obtained in these counts of cell populations within the central nervous system of *Eleutherodactylus* are shown in Tables 1 and 2. As in a previous study on cell populations in the dorsal root ganglia of *Xenopus* (Hughes & Tschumi, 1958) only nucleoli were counted in each section, with the object of minimizing error due to the spread of cells through adjacent sections. In

TABLE 2

Counts of cells in brachial and lumbar ventral horns of E. ricordii. *Two individuals were counted at – 5 days, and also at the adult stage*

	Brachial ventral horn		*Lumbar ventral horn*	
Days before hatching:				
7	654	606	783	704
5	693	666	1,015	806
5	876	687	899	854
3	528	493	598	687
1	553	506	656	654
Adult individuals:				
I	483	414	475	470
II	613	623	557	585

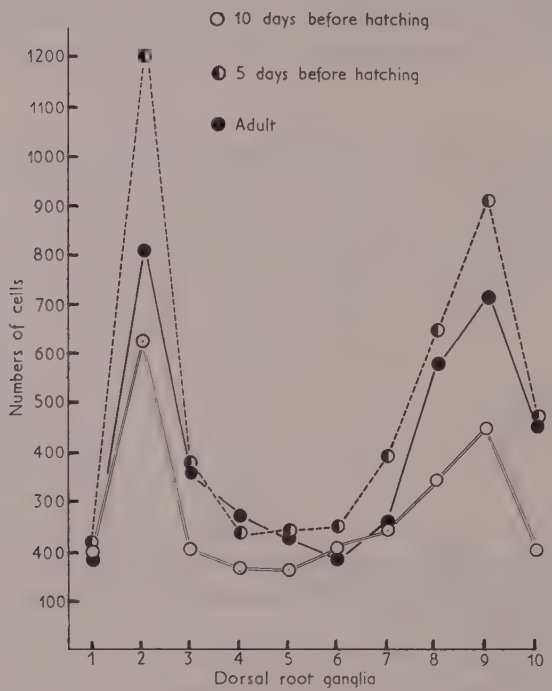
Eleutherodactylus there are ten pairs of dorsal root ganglia belonging to the trunk, including the morphological first pair, the ventral root of which gives off the hypoglossal nerve before joining the brachial plexus.

Table 1 and Text-fig. 3 show that the numbers of cells in the dorsal root ganglia of *Eleutherodactylus* from – 9 days onwards do not greatly change during the remainder of the embryonic period. They reach a maximum between 5 and 7 days before hatching. At first cell production by mitosis more than compensates for the loss of cells by degeneration; after – 5 days mitotic figures disappear from the ganglia while pycnotic nuclei continue to be found until the end of embryonic life. Table 2 and Text-fig. 3 indicate that in the ventral horns also there is a maximum cell population at – 5 days. Here, however, cell degeneration is at first opposed not by mitosis, but by differentiation of fresh ventral horn neuroblasts from the adjacent mantle layer of the cord. Pycnotic nuclei are most frequently seen at the inner border of the ventral horn (Plate, fig. F).

DISCUSSION

The significance of the results which have just been described is not confined to questions of cell numbers. In *E. ricordii* the dorsal root ganglia are first separately recognizable at – 10 days. Throughout the series the numbers of constituent cells are from the first closely similar to those of the adult animal (Text-fig. 4). The main limb ganglia are already larger than are the other members of the series. Yet this stage of development precedes the entry of nerve fibres

into the limbs by one day. Consequently, the general pattern of the spinal ganglia in *Eleutherodactylus* must be attained in the first place independently of any trophic influences derived from the limbs, and with respect to cell numbers the development of the ganglia is a process of self-differentiation.



TEXT-FIG. 4. Average cell numbers in each spinal ganglion in *E. ricordii* at the three stages indicated.

These results may be compared with those obtained for the dorsal root ganglia of the chick in a series of studies by Levi-Montalcini & Levi (1942, 1943) and Hamburger & Levi-Montalcini (1949). In the first two of these papers the 25th was chosen as an example of the lumbar ganglia of the chick. Here, the full number of cells, over 10,000 in all, is attained by about 8 days of incubation, though in one embryo 11,000 were counted on the 5th day (Levi-Montalcini & Levi, 1942). The proportion of differentiated neurones among these steadily rises to nearly 90 per cent. at the end of the incubation period. If, however, the hind limb is removed at the end of the 3rd day, cells degenerate within the lumbar ganglia, so that the total number within the 25th ganglion is then decreased by about two-thirds. A second consequence of the operation is that the number of differentiated cells remains at a low level such as is seen in a normal 6-day embryo. These results have led to the conclusion that in some way the developing limb exerts a trophic influence which not only maintains the cells within the

ganglion, but stimulates their differentiation into mature neurones. This conclusion is supported by comparison within the normal embryo of ganglia at limb and non-limb levels (Hamburger & Levi-Montalcini, 1949). In the latter, from 6 to 9 days of incubation, not only is the mitotic rate at a level lower than in limb ganglia, but also degenerating cells are conspicuous. Thus, as Hamburger (1952, p. 125) says, 'It is of considerable interest to note that the regressive changes produced in limb ganglia as a reaction to limb extirpation are identical with changes which occur in cervical and thoracic ganglia as part of the normal pattern.' However, as Ernst (1926) has observed in a series of normal Amniote embryos, cell degeneration is also to be found within the dorsal root ganglia at limb levels.

The reduction in size of limb ganglia which are prevented by various means from innervating a limb has been observed in a series of studies on various vertebrates (Piatt, 1948; Hughes & Tschumi, 1958), though the effect has nowhere been studied in greater detail than by Levi-Montalcini and her colleagues.

In *Xenopus*, however, before the trophic influence of the limbs is exerted upon the limb ganglia, the effect of intrinsic factors is already recognizable (Hughes & Tschumi, 1958). As is often true, self-differentiation precedes dependent differentiation. The self-differentiating factors in the limb-ganglia of *Xenopus* are in operation before any nerve fibres have entered the limbs; already these ganglia contain a larger number of cells than do the adjacent members of the series, and in them the maximum cell size is restricted to a level below that seen elsewhere among the dorsal root ganglia.

In *Eleutherodactylus* the relative differences in cell number among the dorsal root ganglia at limb and non-limb levels are already evident as soon as the ganglia are separately recognizable and a day before any nerve fibres enter the limb-buds. Here neither differential rates of mitosis nor selective cell-degeneration play any part in shaping the final relative sizes of the spinal ganglia. Within them the mitotic rate never rises above a relatively low level, and cell degeneration is found to an equal extent throughout the series of ganglia. Cells degenerate both in the limb ganglia and in the ventral horns of *Eleutherodactylus* notwithstanding any trophic influences which emanate from the limbs. Glücksmann (1951), in a valuable and comprehensive review, has shown that cell death is associated with many aspects of vertebrate development in a range of tissues and at various stages of differentiation. Cell degeneration must constitute an element in several distinct aspects of the development of the nervous system, and these must vary in importance from one example to another.

Eleutherodactylus is admittedly something of a special case. Its ontogeny is adapted not only to an embryonic mode of development, but also to the minute size of the future adult in such respects as the small number of cells in dorsal root ganglia and ventral horns. Furthermore, development in *E. ricordii* is particularly rapid, as has already been noted. In other vertebrates, as far as is known, the number of cells in the dorsal root ganglia is mainly controlled by peripheral

factors, such as the formative stimuli which emanate from the limb-buds. Such dependent differentiation must need a minimum time-scale on which to operate. To determine how far the rapid development of *E. ricordii* restricts the scope of these influences, however, demands the crucial experiment of limb ablation. The relative importance of each of Roux' complex components in various patterns of ontogeny would be an important topic in a comparative neuro-embryology.

SUMMARY

1. In the West Indian Anuran genus *Eleutherodactylus* the larval stage is wholly suppressed. As in other systems of organs there are features in the spinal cord which are correlated with this embryonic type of development.

2. The cord in the trunk region resembles more that of a chick embryo than that of a larval Amphibian. Within the thick neuroepithelium mitoses in the ependymal zone persist much longer than in larval forms. There is a relatively thin layer of white matter.

3. The Rohon–Beard system of sensory neurones persists, but does not precede the development of the dorsal root ganglia. In the trunk there are no conspicuous primary motor-cells as in larval Amphibia.

4. The numbers of cells within the dorsal root ganglia and ventral motor horns have been counted both in the embryo and in the adult. From the first, the numbers of cells present in both are similar to those in the adult. During development, increase by mitosis is opposed by cell degeneration. Pycnotic nuclei are conspicuous in both spinal ganglia and ventral horns in the embryo.

5. As soon as the spinal ganglia are recognizable, their relative sizes are similar to those of the adult. The ganglia at limb levels are already larger than those elsewhere a day before nerve fibres first enter the limb-buds.

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EXPLANATION OF PLATE

All figures are of sections through embryos of *E. ricordii.*

FIGS. A, B. and C. Transverse sections of spinal cord in trunk region. A. −11 days. Haematoxylin and eosin. ×140. B. −9 days, showing early stage of lumbar ventral horn (cf. Fig. E). Bodian. ×150. C. −7 days, through lumbar ventral horn at second stage. Haematoxylin and eosin. ×140.

FIG. D. Transverse section through half of spinal cord at −9 days, to show Rohon-Beard cell (extreme top of figure) with axon root. Central canal at left margin. Bodian. ×640.

FIG. E. Transverse section through part of spinal cord at −9 days. Same section as in Fig. B showing lower half of right-hand ventral horn, below which are the first fibres of the longitudinal motor column. Many neuroblasts in ventral horn already bipolar. ×640.

FIG. F. Transverse section through part of spinal cord at −5 days to show lumbar ventral horn

and adjacent mantle layer to left. End stages of cell degeneration between them (arrow). Haematoxylin and eosin. ×640.

FIG. G. Transverse section through dorso-lateral part of spinal cord at −7 days. The section is taken through the trunk region, near the junction with the tail, and shows a Rohon–Beard cell still in the original position. Haematoxylin and eosin. ×600.

FIG. H. Transverse section through spinal cord in tail region of same embryo as in Fig. G to show Rohon–Beard cell in dorsal position. Haematoxylin and eosin. ×640.

FIG. I. Part of horizontal section through dorsal region of spinal cord at −6 days. The axis of the embryo lies across the picture. At upper and lower margins are shown the dorsal funiculi on each side; inwards from each are a row of Rohon–Beard cells. One in upper part of picture (arrow) receives an afferent fibre from the skin. Rohon–Beard cell at lower margin gives off a longitudinal fibre. Bodian. ×650.

FIG. J. Part of section through hind limb-bud at −9 days, showing the first nerve fibres which have just entered the base of the limb. Bodian. ×620.

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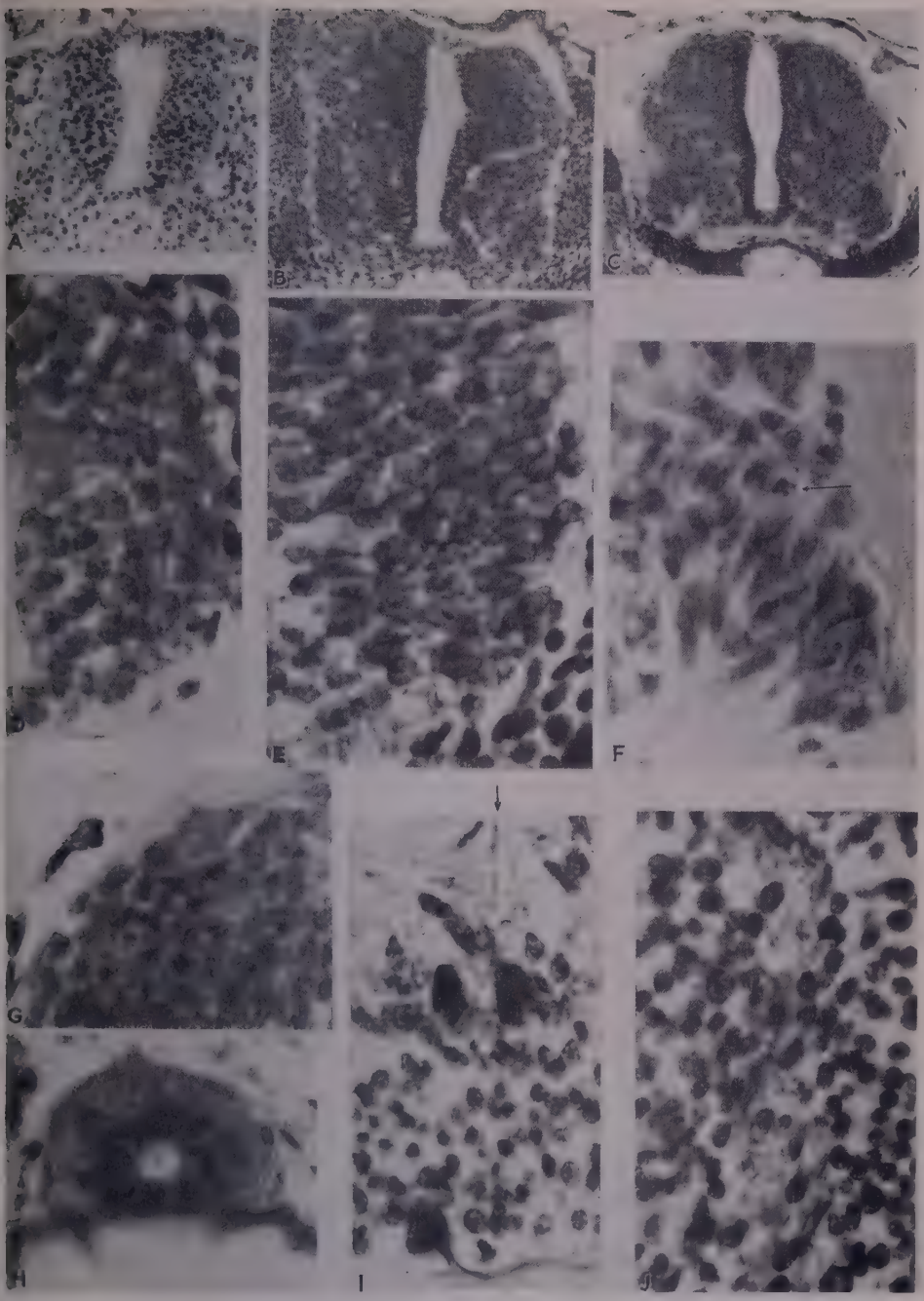
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Genetical and Developmental Studies on Droopy-eared Mice

by G. A. CURRY[1]

From the Institute of Animal Genetics, Edinburgh

WITH TWO PLATES

GENETICS

DROOPY-EAR, designated *de*, arose spontaneously in Dr. Falconer's J stock of mice in Edinburgh, and appeared later when the stock was inbred in 1952. The mutant is named after its most striking phenotypic characteristic; the ears are set low down the sides of the head, and the pinnae project laterally. On a genetic background (a^t and A^w) which causes the belly hair to be lighter than the back hair, the belly hair comes up farther round the sides of the body and face. These characteristics of droopy-eared mice are always distinctive. Droopy-eared mice tend to be smaller than their full sibs, but they breed well, except for some females who are bad mothers.

Classification may be done at birth by the position of the ears, and the outline of the scapula and acromion which can be seen through the skin. In droopy-eared mice these are always reduced in size, and the acromion is abnormal in its shape.

TABLE 1

Mating types	+	*de*	*Total*	χ^2
de/*de* × *de*/*de*	0	63	63	..
de/*de* × +/+	216	0	216	..
+/*de* × +/*de*	116	35	151	0·32
+/*de* × *de*/*de*	124	106	230	1·14

Droopy-ear is due to a single, recessive, fully penetrant gene, as is shown by the results from four different types of matings given in Table 1. The very slight deficiency of droopy-eared mice in the segregating families is not significant. However, of the 204 *de* mice listed above 37 died before they were weaned, while only 9 of the 456 normals died; these figures do not include the deaths of whole families which had been eaten by bad mothers. This difference is significant, and

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shows that droopy-ear is less viable than normals during the first few weeks of post-natal life.

Tests for linkage and independence were also carried out with the linkage stocks designed by Carter & Falconer (1951). It was found that droopy-ear

TABLE 2

Analysis of linkage of droopy-ear with Varitint-waddler

Mating types	Phenotype of progeny				Total	D.	I.	Recomb. (%)		χ^2
	+Va	++	deVa	de+				p	se	
(*a*) Backcross. Repulsion. *de*+/*de*+ × *de*+/+*Va* .	48	22	19	14	133	−160	532	31±4·3		30
(*b*) Backcross. Coupling. *de*+/*de*+ × *deVa*/++ .	13	46	35	13	108	−110	432	25±4·8		28
Combining these figures					241	−270	964	27±3·2		71

Key: p = recombination factor; *se* = standard error.

TABLE 3

Independence of droopy-ear

Mutant M or m	Linkage group	Mating type	Phenotype of progeny				Total	Recombination (%)	
			$+^{de}M$	$+^{de}m$	*deM*	*dem*		p	se
Wh	XI	BR	27	35	20	29	111	50±4·7	
T	IX	BR	27	26	20	20	88	50±5·2	
N	VI	BR	28	27	25	14	94	55±5·2	
Re	VII	BR	22	29	18	23	92	48±4·2	
Sd	V	BR	25	38	10	25	98	50±5·2	
s	III	IR	65	27	14	4	110	44±7·2	
wa	VII	IR	79	13	15	5	112	59±7·1	
ln^2	XIII	IR	74	18	14	5	111	58±7·1	
b	VIII	IR	56	12	13	4	86	45±8·0	
Ra	V	BR	34	37	19	22	112	50±4·7	
p	I	IR	93	22	20	7	136	55±6·4	
se	II	IR	90	25	19	8	136	55±6·4	
fz	XIII	IR	83	32	21	6	136	42±6·4	
v	X	IR	81	32	22	4	133	41±6·4	
ru	XII	IR	87	33	23	7	150	37±6·2	
je	XII	IR	89	30	23	2	144	40±6·3	
f	XIV	IR	87	29	19	7	142	50±6·3	
c^e	I	IR	91	29	21	3	144	46±6·3	
W^v	III	BR	26	29	15	18	88	50±5·3	
♂		BR	63	47	47	38	195	52±3·6	

Key: *M* or *m* = mutant gene; B = backcross; I = intercross; R = repulsion; ♂ treated as dominant.

and Varitint-waddler were not segregating independently of each other; see Table 2 (*a*). Further matings were set up with droopy-ear and Varitint in the opposite phase, and the results are shown in Table 2 (*b*). Combining these two sets of information, the results show that droopy-ear and Varitint are linked by

a recombination factor of $27 \pm 3{\cdot}2$. These two mutants have now been assigned to a new linkage group, number 16. Droopy-ear was found to segregate independently of the other twenty genes tested; similarly it was found to segregate independently of sex (Table 3).

Thus droopy-ear, with its clear penetrance and distinctive manifestation at birth even in the presence of other diverse genes, shows great promise as a genetic marker.

ANATOMY

Methods

A complete examination of the droopy-ear skeleton was made and compared with that of the normal mouse. Skeletons of contemporary full sibs, one mutant and one normal, from 11 days to 7 months old were prepared in one of four ways: by papain digestion (Luther, 1949); by boiling and then cleaning off the flesh with forceps; by partial papain digestion, followed by careful cleaning with forceps, in order to keep the epiphyses in position; or by the alizarin clearance technique (Johnson, 1933). In all over eighty skeletons were obtained.

This description of the adult anatomy is based on ten such pairs of 6-week-old mice, and the drawings as far as possible are of the most characteristic bone of the group. These drawings were made with the aid of the camera lucida at an original magnification of $\times 11$; the anglicized names (Greene, 1935) have been used wherever possible, and others have been adopted from Bateman (1954). The parts labelled are those mentioned in the text, and the labelling is only done on the normal where the equivalent part on the droopy-eared bone is not clear. The names on the bones in inverted commas are my own for the purpose of making the description easier. The term 'poor definition' has been used to describe bones which lack characteristic sharp angles, or clean smooth curves (cf. Text-fig. 11).

Results

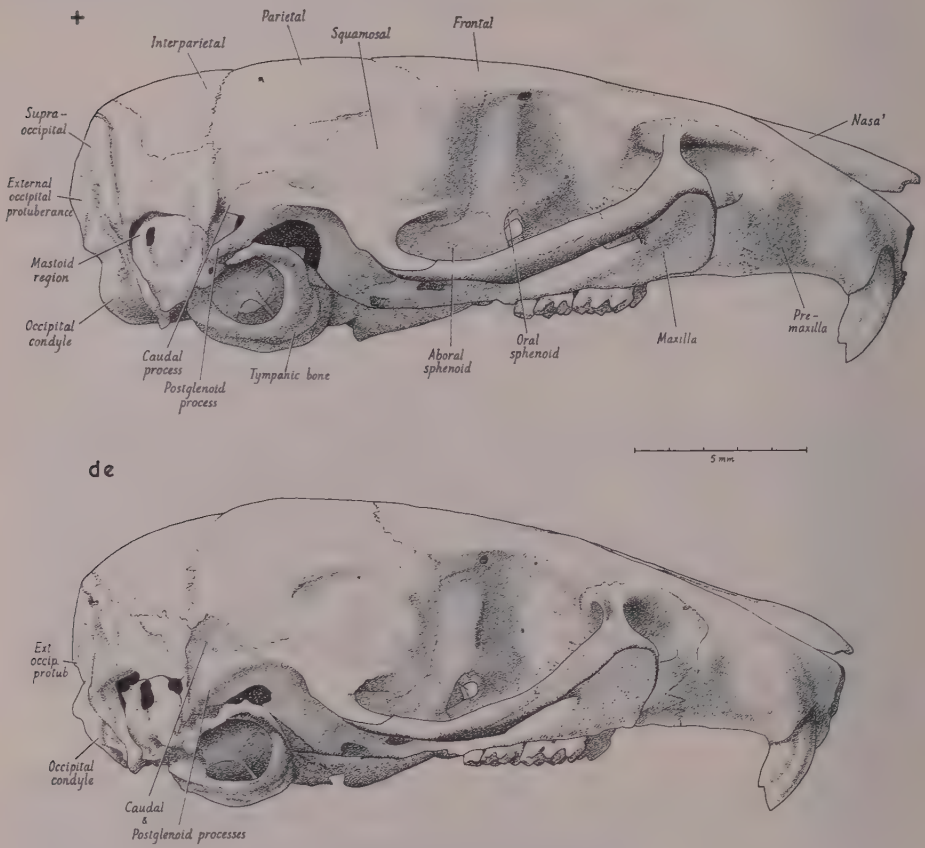
The skeleton was found to be generally affected, while the posterior region of the skull, the neck, and the shoulder girdle showed more specific abnormalities.

Skull (Text-fig. 1). The most general abnormality is a transient immaturity and a slight hydrocephaly, which is clear at 6 weeks, but is completely undiscernible in mice of 3–7 months old.

The nasal, vomer, and premaxilla show the closest approximation to the normal, while the rest of the skull is short and deep. In the posterior region there is a displacement of bones; the supraoccipital is vertical, the height of the foramen magnum is small, and the ear vesicles are drawn centrally and thus face slightly ventral. This is best seen in the drawing of the alizarin preparation of a 3-week-old skull (Text-fig. 23).

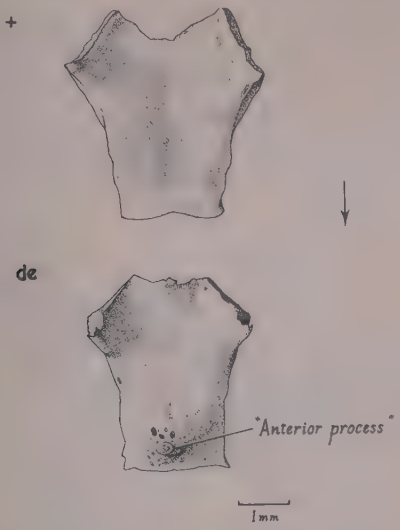
The more specific abnormalities of the individual parts of the skull are seen in the squamosum (Text-fig. 1), where the glenoid and caudal processes are fused into one heavy mass of bone. The basioccipital (Text-fig. 2) varies especially at

the occipital border, which is generally asymmetrical, and a 'process' is often present on the anterior cranial surface. Posteriorly there is much pitting indicative of abnormal osteoclastic activity. Fusion to the lateral occipital has not occurred, and so, to make the drawings of the normal and droopy-ear compar-

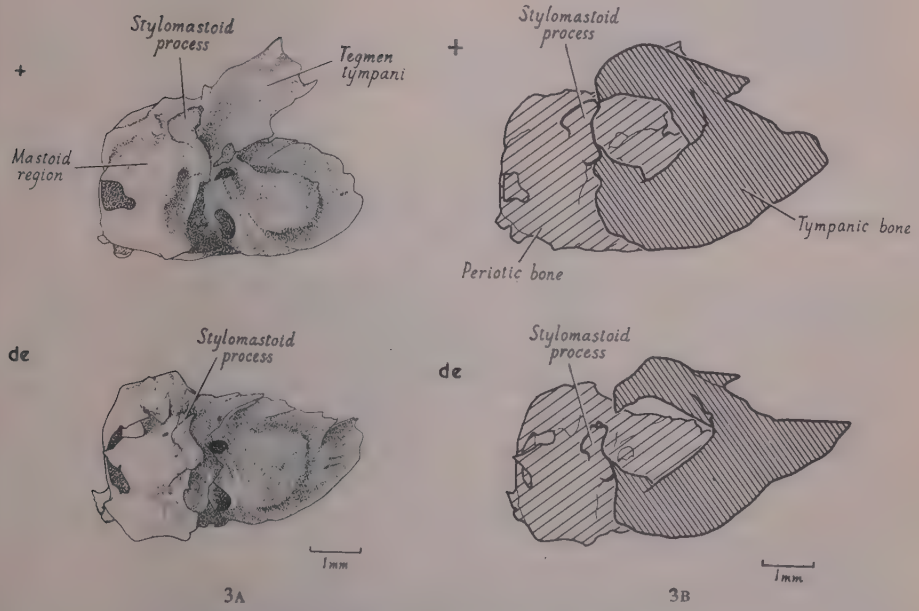


TEXT-FIG. 1. Skull. Lateral aspect.

able, both were drawn at 4 weeks, before fusion normally takes place. The ear ossicles were found to be normal, except for a few rare cases where they were twisted, and carried small bony excrescences. The periotic bone (Text-fig. 3A) is rugged, the tegmen tympani is entirely missing, and the stylomastoid process is small and malformed. As a result the periotic and tympanic bones are held together very precariously, and the middle ear-drum has a non-bony region all down the dorso-lateral side (Text-fig. 3B). It is to be expected that such a condition might affect the hearing ability of the mice. This was investigated, and the mice were found not to be deaf.



TEXT-FIG. 2. Basioccipital (4 weeks old). Cranial surface. The arrow here and in all other text-figures indicates the anterior direction.

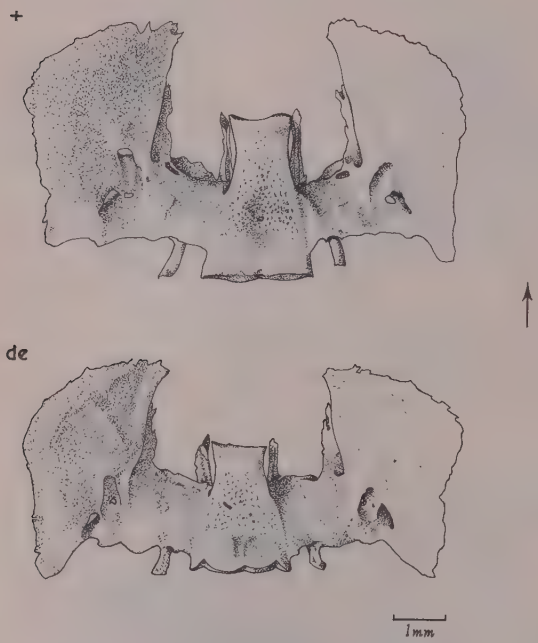


3A 3B

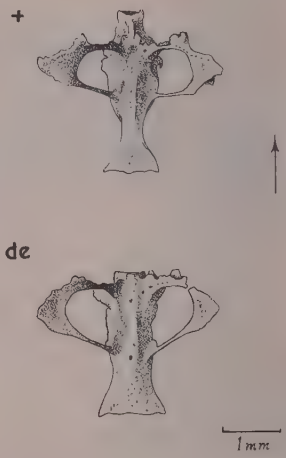
TEXT-FIG. 3A. Right periotic bone. Lateral aspect.

TEXT-FIG. 3B. Right auditory capsule. Diagram to illustrate the non-bony region down the dorso-lateral side of the middle ear in *de*.

The sphenoids (Text-figs. 4 and 5) are both reduced in length, and rather heavy in structure, and they also show irregular pitting, similar to that seen on the basi-occipital. On the ethmoid, the ethmoidal laminae are heavy and foreshortened, but the cranial aspect is quite normal. The maxilla is wide, mainly as a result of the increased angle between the zygomatic process and the central part of the bone. The bone itself is slightly wide, and the articulation surface with the frontal



TEXT-FIG. 4. Aboral sphenoid. Cranial surface.

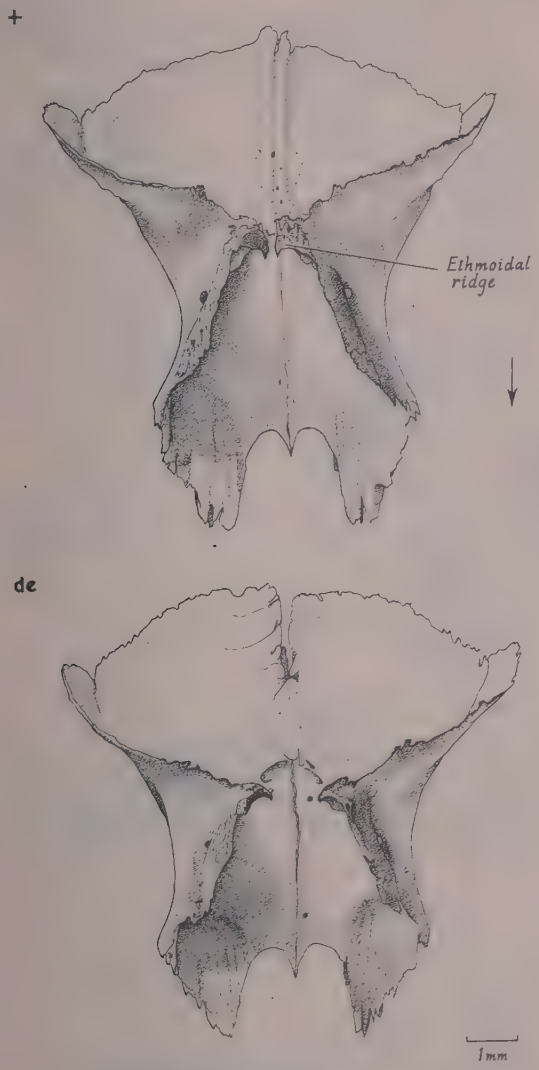


TEXT-FIG. 5. Oral sphenoid. Cranial surface. The aboral and oral sphenoids were chosen from the same mouse in order to show the total reduction in length of the sphenoid region.

is displaced laterally; this results from the considerable increase in width of the frontals (Text-fig. 6). The ethmoidal crests of the maxilla do not meet in the mid-line. The frontals are a further example of abnormal fusion times. Normal fusion occurs at 4 weeks, but in droopy-ear if fusion has occurred at 6 weeks it is still incomplete (Text-fig. 6). The parietal shows the general characteristics of being short and broad, while the interparietal is extremely long antero-posteriorly; this will be discussed later. On the mandible the mandibular head is displaced on the condyloid process. The angular process is short, and the maseoteric ridge poorly defined, as are many droopy-eared bones.

Vertebral column. In general the vertebrae are short antero-posteriorly, especially in the cervical and lumbar regions. The atlas and epistropheus (Text-figs. 7 and 8) show reciprocal abnormalities. They are malformed, and the dens is in the position of the centrum of the atlas as in ancestral forms. In one case the atlas was found to be fused to the occipital region. The ribs and sternum show no

striking abnormalities, but, typical of so much of the droopy-ear skeleton, they show poor definition.



TEXT-FIG. 6. Frontals. Cranial surface.

Pectoral girdle and forelimb. Here the specific abnormalities are found in the clavicle and scapula, and the acromion is greatly reduced. The clavicle (Text-fig. 9) has a foreshortened shaft, but in the region of the acromial extremity it is extended to form an 'extra' acromial extremity, apparently compensating for the reduction of the acromion. The scapula (Text-figs. 10A and 10B) is small, and the

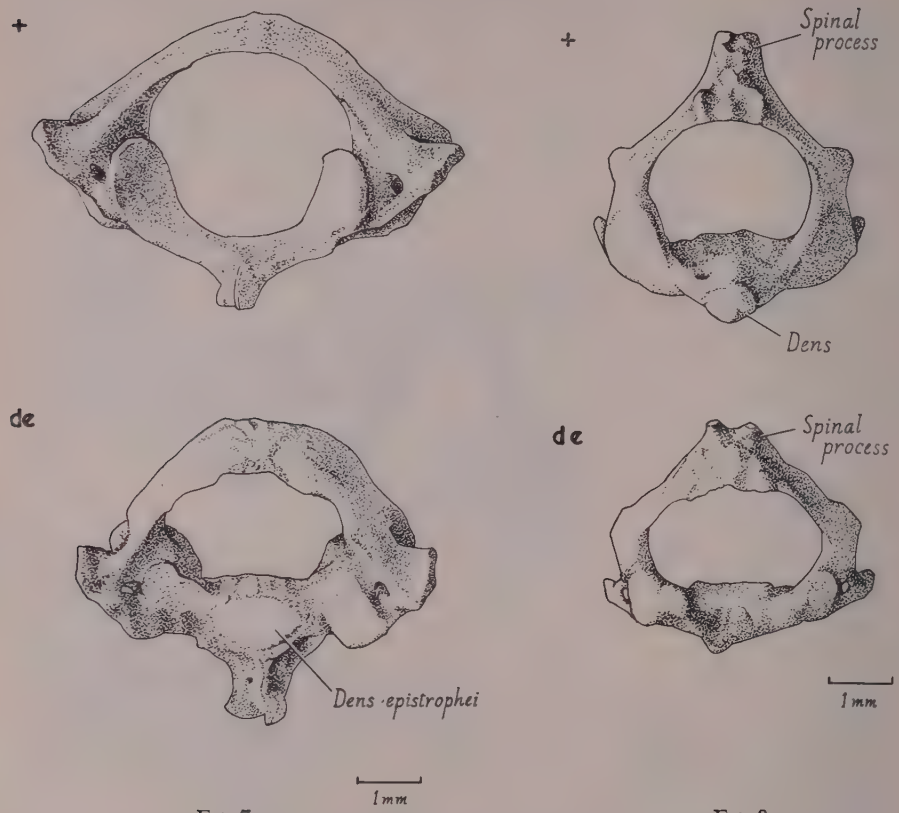
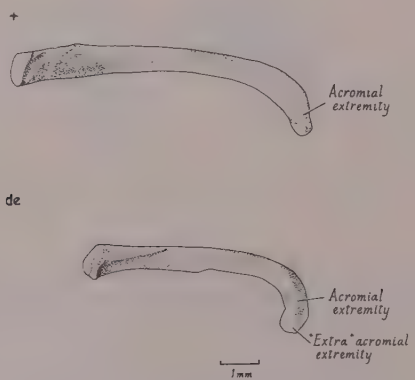


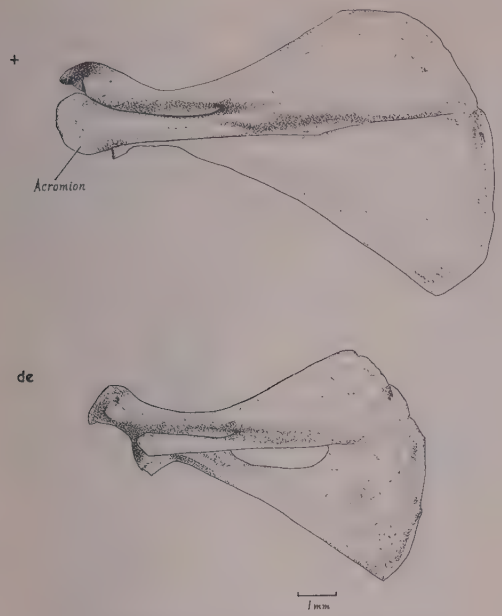
FIG. 7 FIG. 8

TEXT-FIG. 7. Atlas. Caudal aspect.

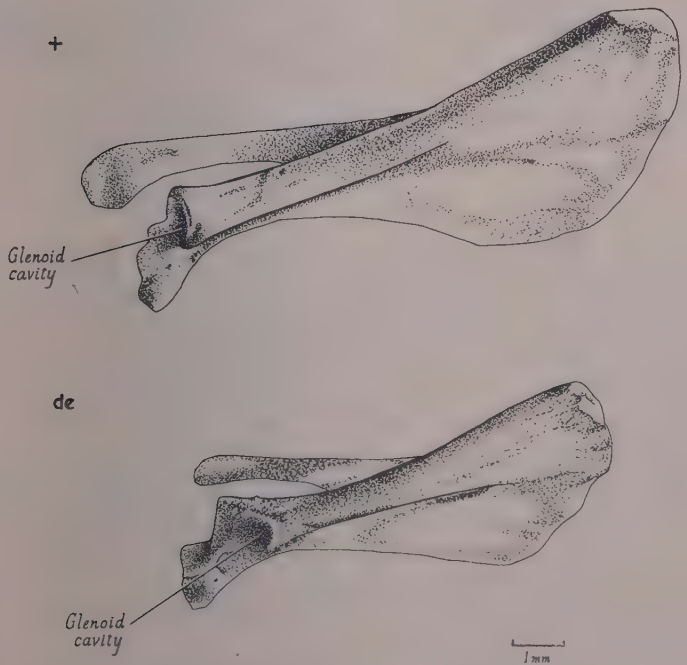
TEXT-FIG. 8. Epistropheus (axis). Cranial aspect. The atlas and epistropheus were chosen from the same mouse, to show their relationship to each other.



TEXT-FIG. 9. Left clavicle. Anterior aspect.



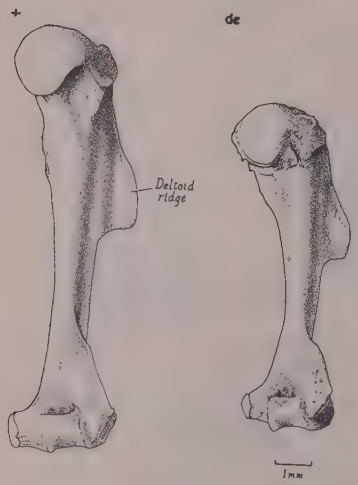
TEXT-FIG. 10A. Left scapula. Lateral aspect.



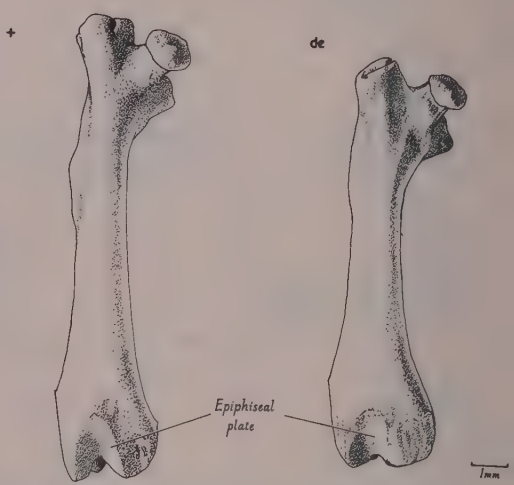
TEXT-FIG. 10B. Left scapula. Inferior aspect.

acromion short and malformed. In the mice examined 70 per cent. had a hole in the posterior part of either or both of their scapulae. This hole was of varying sizes but it never broke the posterior border of the scapula. The angle of the surface of the glenoid cavity was found to vary through 90°, so that in some cases the clavicle played a large part in the articulation of the humerus, while in the other extreme case when the glenoid cavity was ventral (Text-fig. 10B) it provided the only articulating surface for the head of the humerus.

These skeletal changes might well be expected to be accompanied by changes



TEXT-FIG. 11. Right humerus. Posterior aspect.



TEXT-FIG. 12. Right femur. Dorsal aspect. All the epiphyses have been removed to show the flattened distal epiphyseal plate in *de*.

in musculature. The musculature of the neck and shoulder girdle was examined and the following changes were found. The acromiotrapezius and spinotrapezius are two superficial muscles, arising on the vertebral column, and inserting on the scapula (Greene, 1935). In droopy-ear both these muscles are very small; they arise normally, but there is scarcely any insertion on to the acromion or scapula, presumably because the bone is so reduced in this region that the muscles can have very little on which to pull. Many of the fibres of the two muscles seem to become interlocking in the region of the scapula. The chief effect of this is to cause the mice to be round-shouldered. The deeper muscles of the neck, shoulder, and forearm were also examined. The only differences from normal were that a greater proportion of the acromio-deltoideus, which arises from the clavicle and acromion, came from the clavicle; and that the levator claviculae was inserted more centrally on the clavicle.

The humerus (Text-fig. 11) is immature and the shaft slightly curved, and the

5584.7 E

TABLE 4

Normal					*Droopy-ear*				
Bone length in mm.			*% of lower jaw length*		*Bone length in mm.*			*% of lower jaw length*	
Sphenoids	*Interp.*	*Lower jaw*	*Sphenoids*	*Interp.*	*Sphenoids*	*Interp.*	*Lower jaw*	*Sphenoids*	*Interp.*
7·0	5·0	13·5	51·9	37·1	6·0	5·5	13·5	44·5	40·8
6·5	4·0	13·0	50·0	30·8	5·5	6·0	13·0	42·3	46·2
7·0	4·5	13·0	53·9	34·2	6·5	6·0	13·5	48·2	44·5
7·0	4·5	13·0	53·9	34·2	6·0	5·5	12·5	48·0	44·1
8·0	4·5	14·5	55·3	31·0	6·0	5·0	12·5	48·0	40·0
8·0	5·0	15·5	51·7	32·3	6·0	5·0	13·5	44·5	37·1
8·5	4·5	15·0	56·5	30·0	6·0	5·0	13·0	46·2	38·2
7·5	5·0	15·5	48·4	32·3	6·5	5·0	13·0	50·0	38·4
			Total 421·7	261·9	6·5	5·5	14·0	46·6	39·4
					6·5	6·0	14·5	44·9	41·5
					7·0	6·0	15·0	46·7	40·0
					7·5	6·0	15·0	50·0	40·0
					7·0	5·5	14·0	50·0	39·4
					6·5	5·5	15·0	43·4	36·7
								Total 653·3	566·3
			Mean 52·7	32·7				Mean 46·6	40·5

The difference between the sphenoid percentages in normal and droopy-ear are significant: $t = 5{\cdot}27$.
The difference between the interparietal percentages in normal and droopy-ear are also significant: $t = 5{\cdot}82$.

proximal epiphyses deformed. The radius and ulna are twisted over each other, so that the articular surface for the humerus is out of alignment. The olecranon process and olecranon are poorly developed. The wrist bones are poorly defined, so that articulations are loose. There is slight reduction in length of the metacarpals and a lesser degree of reduction in the length of the phalanges.

The pelvic girdle and hind limb. The bones of the pelvic girdle are poorly defined, and the ilium is not fused to the ischium and pubis until 8 weeks or later; normally this fusion is complete at 6 weeks. The hind limb shows abnormalities similar to many of those in the forelimb. The femur (Text-fig. 12) has immature proportions, being short and thick. The trochanter tertius is very poorly developed. The tibia and fibula are slightly twisted, and the intraosseous groove is very shallow. Again there is a loose articulation in the ankle due to the poor definition of the tarsal bones, and the chief region of foreshortening is the metatarsals.

The adult skeletal anatomy reveals that the general characteristic of droopy-ear is immaturity and loss of definition, accompanied by some peculiar fusion times. More specific abnormalities occur in the occipital region, the neck, and the pectoral girdle. There is one other matter which it is convenient to raise at this point. The cartilage bones have been shown to be generally shorter, while some membrane bones are increased in size, such as the interparietal, the squamosum, and the clavicle. In order to test if this change in size was absolute, the length of the interparietal antero-posteriorly and of the sphenoids was measured and expressed as percentages of lower jaw length. The results are shown in Table 4. In both cases the proportions of the normal and droopy-eared bones are significantly different. So in droopy-ear the sphenoids are disproportionally reduced, the basioccipital is drawn anteriorly, the supraoccipitals become vertical, and the interparietal enlarges in the peculiarly convenient manner of membrane bones to fill the gap which is left (Murray, 1936). The enlargement of the squamosum is not so easily explained, since it has occurred at birth before one is aware that there was any gap to be filled; it may, however, be linked with the absence of the tegmen tympani. A similar property in the clavicle might account for its compensating for the loss of the tip of the acromion.

DEVELOPMENT

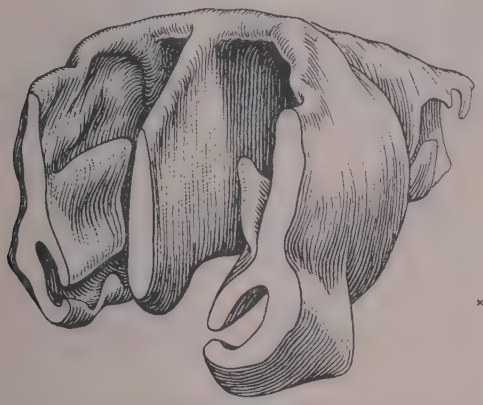
Methods

The development of the cartilage was studied in sectioned material and by means of methylene blue transparencies, whereby the cartilage is stained blue while the remaining tissue is pale blue or colourless, and the development of the osseous skeleton by the alizarin clearance technique, in which the osseous tissue takes up the dye, the rest remaining colourless.

Embryos were aged according to the description and classification made by Grüneberg (1943). The comparative description is based on the study of contemporary full sibs obtained from heterozygous matings.

The drawings of cleared preparations were made with the aid of a camera lucida, but these were not always as accurate as could be desired owing to the vague forms and the depth of focus required. Therefore the drawings should be considered as diagrams showing the areas of cartilage and bone formation. The shading indicates the intensity of stain, and should only be compared within age groups, since each age group was stained at a different time, and the degrees of staining between groups may not be comparable with one another. The labelling in inverted commas indicates that the cartilage has been named according to the bone which is to form in that area.

When the diagram of the chondrocranium of a normal 15-day-old embryo had been completed, it was noticed that the anterior region was not in accordance



× 45

TEXT-FIG. 13. Fifteen-day embryo. The cartilaginous form of the skull anterior to the eye, reconstructed from serial sections.

with that drawn by Grüneberg (1953), in which there was a cartilaginous 'projection', labelled planum antorbitale. In this work the planum antorbitale has been interpreted not as a projection, but as a folded sheet of cartilage seen in the foreshortened view, which, when seen from other angles, was a thin sheet encasing the sides and part of the floor of the nasal region. Serial transverse sections confirmed this interpretation. A perspective drawing of this region, built up as a reconstruction from the serial sections, was made (Text-fig. 13). It shows the cartilage from the anterior region of the eye to the tip of the nose as continuous.

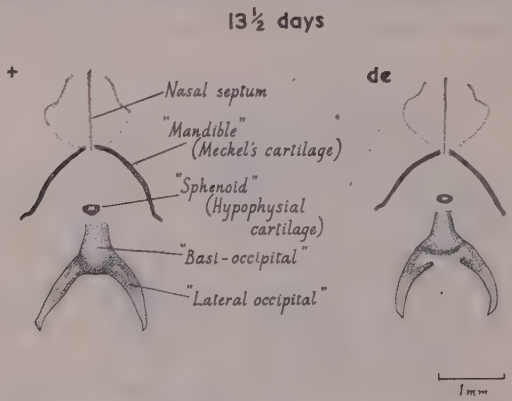
Serial reconstructions were also found very helpful in interpreting other obscurities in the occipital region.

Results

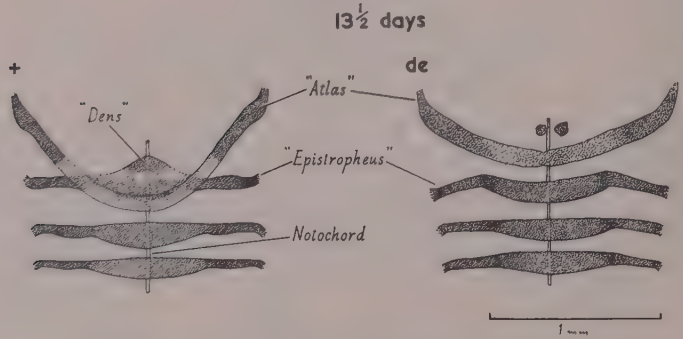
At 11 days the region of the occiput, atlas, and epistropheus show mesenchymal condensations which in droopy-ear appear to be composed of cells

of irregular shape, but the difference from the normal is not enough to be diagnostic.

At 12 days droopy-eared embryos can be recognized with certainty. In the normal the posterior region of the basioccipital is rounded, and the cells are arranged in regular manner. In droopy-ear the basioccipital is thin, and the posterior border flattened and extended ventrally (Plate 1, figs. A and C). The arrangement of the cells is irregular and their density greater (Plate 1, figs. B and D). The mesenchymal condensation of the scapula is now recognizable, but in droopy-ear it never reaches the same maximum thickness as the normal.



TEXT-FIG. 14. Dorsal view of the chondrocranium.

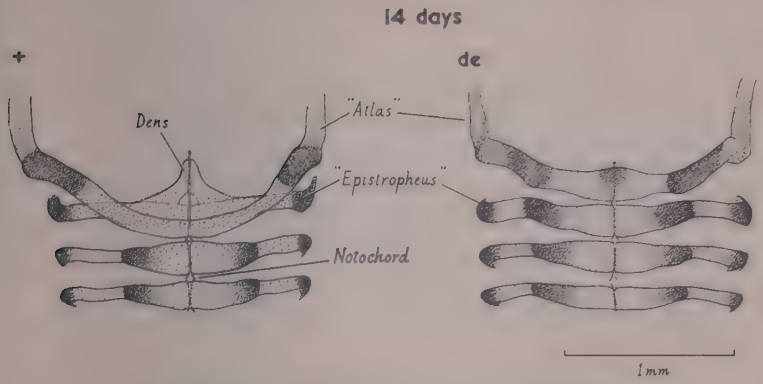


TEXT-FIG. 15. Ventral view of the upper cervical vertebrae.

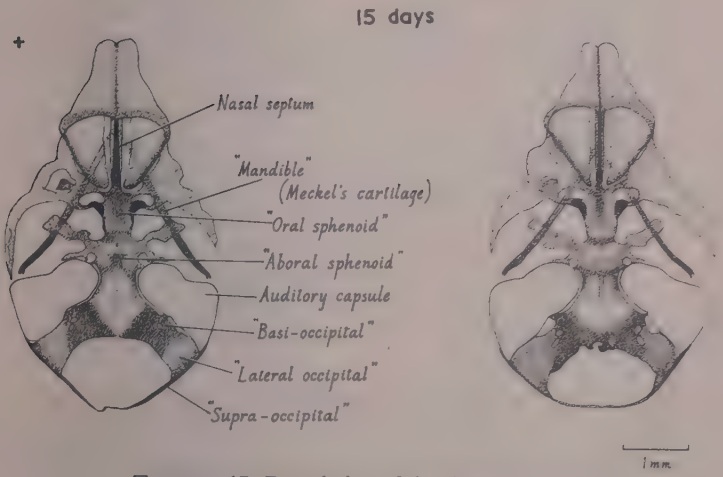
In the 13-day-old embryos the conditions described above are exaggerated (Plate 1, figs. E and F). In order to draw the chondrocranium and cervical region in the methylene blue transparencies the specimens had to be decapitated. In droopy-ear this necessitated breaking a cartilaginous connexion between these two regions. The chondrocranium and cervical region in Text-figs. 14 and 15 are from the same embryo, and it would appear that the two cartilaginous lumps anterior to the atlas have been torn from the space between the lateral occipitals.

These two lumps are representative of the dens. The atlas and epistropheus are far apart, and the cartilages of these, and the next three cervical vertebrae, are short.

In the scapula chondrification has now started, but in the droopy-ear it is thin; the hole, if present, is an area entirely lacking any mesenchymal condensation or



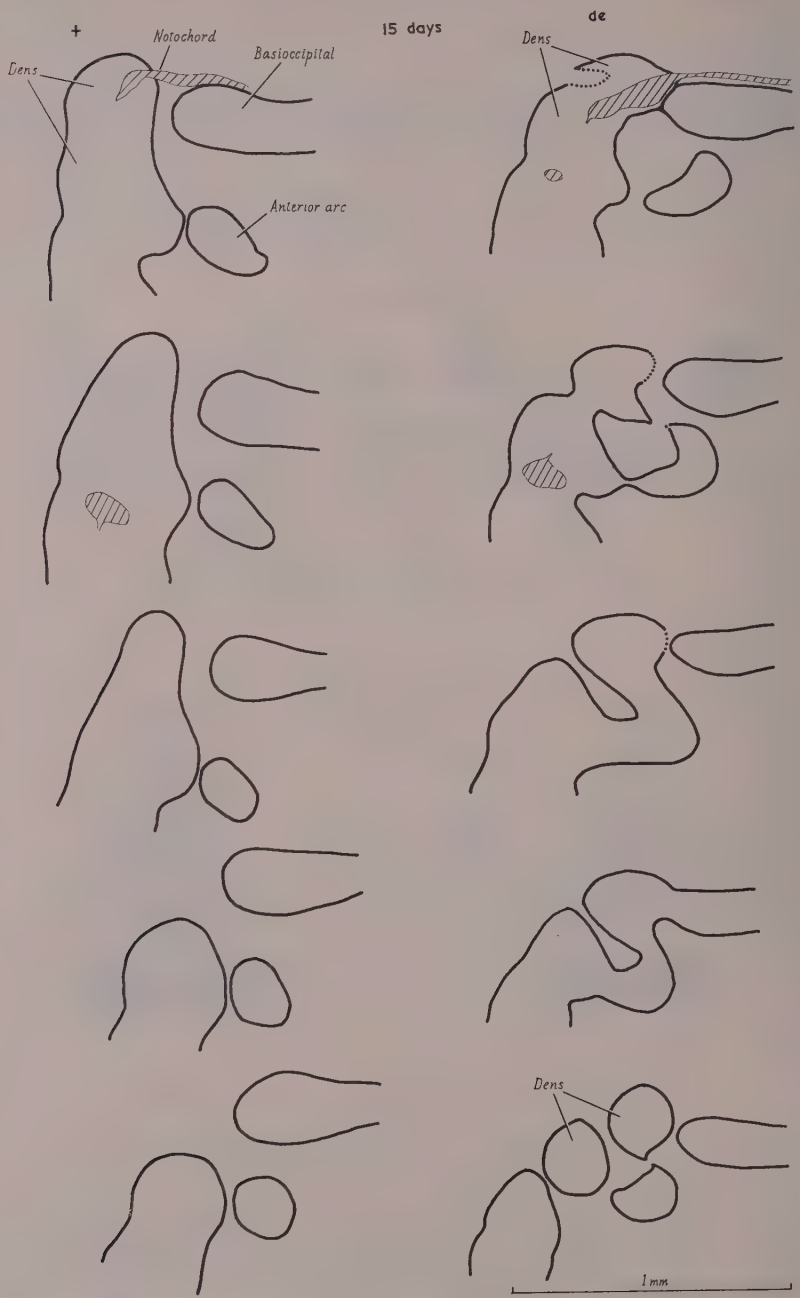
TEXT-FIG. 16. Ventral view of the upper cervical vertebrae.



TEXT-FIG. 17. Dorsal view of the chondrocranium.

cartilage cells (Plate 2, figs. M and N). In the methylene blue preparations (Text-fig. 19) it can be seen that the scapula and forelimb are small; the scapula is abnormal in its shape and staining property, and the acromion if present has not stained at all.

No abnormalities are seen in the pelvic girdle, but the condition in the hind



TEXT-FIG. 18. Outline drawings, at intervals of 90 μ of midsagittal sections of the basicranial and upper cervical region, showing the splitting of the dens in *de*.

limb is similar to that in the forelimb. The whole rudiment is small and ossification is retarded in the femur and tibio-fibula at 13 and 14 days, but this retardation is transitory as at 17 days ossification is proceeding normally.

In the 14-day-old embryo it is normal for the anterior arc of the atlas to be fused to the dens epistrophei (Plate 1, figs. G and H). In droopy-ear the dens is split and the anterior part may be fused to the basioccipital. These connexions do not usually ossify, as generally the only abnormality found in this region in the adult is that the dens is fused to the atlas; but in one case the whole of this region was found fused together.

The difference in cell structure of the basioccipital in normal and droopy-ear is shown in Plate 1, figs. I and J. In droopy-ear the cells are less clearly defined, and the cell density greater. In the methylene blue preparations the basioccipital showed no signs of an ossification centre as is normal at this stage (Grüneberg, 1953). In Text-fig. 16 the relative positions of the atlas and epistropheus are abnormal and the dens is incorporated in the atlas. The condition in the droopy-ear scapula is similar to that of the basioccipital: it is thin (Plate 2, figs. O and P) and the cell size and shape are abnormal and the alignment irregular (Plate 2, figs. Q and R).

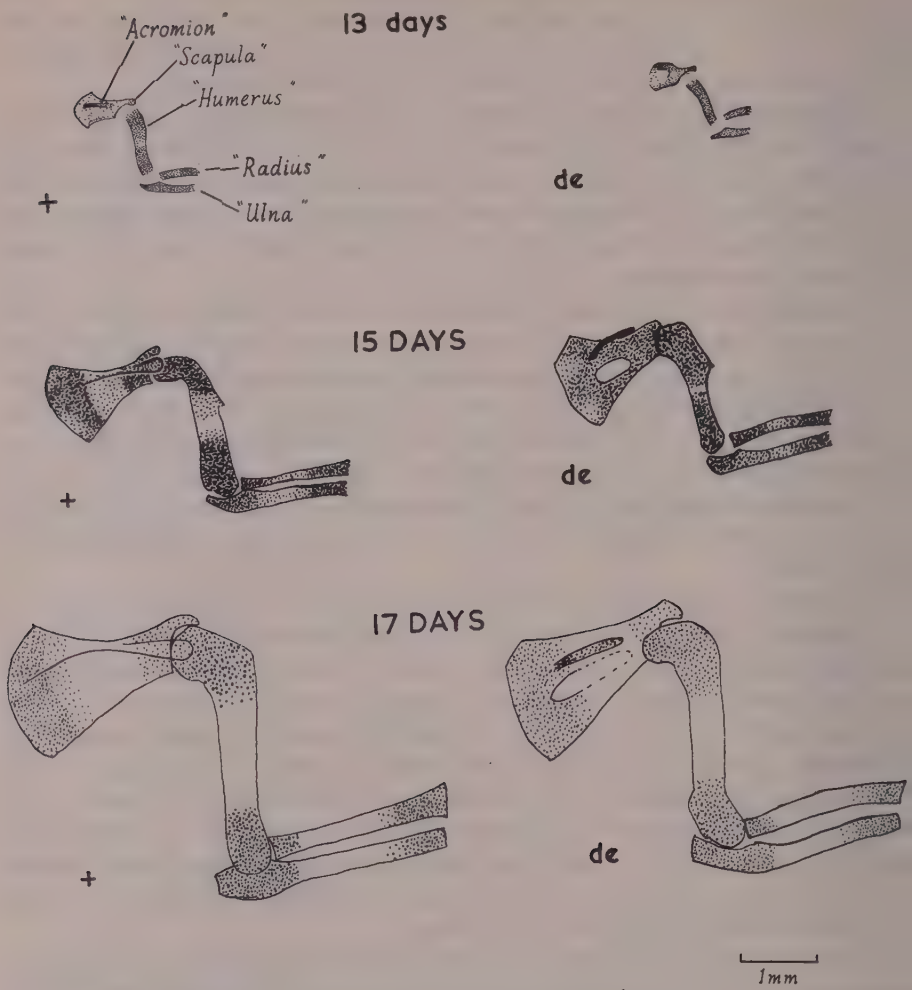
Normally in the 15-day-old embryo the ossification of the posterior region of the basioccipital is quite far advanced, and the anterior arc of the atlas is no longer fused to the dens epistrophei. In droopy-ear both the sectioned material and the methylene blue preparations (Text-fig. 17) show that ossification of the basioccipital is retarded. In the chondrocranium the sphenoid region has stained differently. The anterior arc of the atlas still has a tenuous connexion with the epistropheus and may also be connected to the basioccipital. Camera lucida drawings of the outlines of the cartilages in this area illustrate this point (Text-fig. 18). If further drawings had been done, the anterior portion of the dens would have been shown to be incorporated into the basioccipital, and the posterior portion fused with the anterior of the atlas.

In the scapula the development is still retarded (Text-fig. 19). The droopy-ear acromion now stains very darkly, while that in the normal is losing this staining ability as the cartilage hypertrophies. The ossification of the forelimb is also retarded.

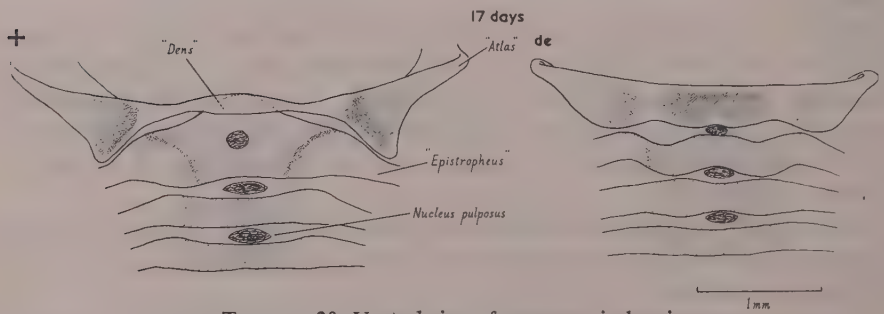
In the 16-day-old droopy-eared embryo the sectioned material shows a double thickening of the notochord in the dens. Normally the notochord has almost disappeared at this stage (Plate 2, figs. K and L). The cleared material showed that in droopy-ear the dens is incorporated into the atlas (Text-fig. 20) and an intervertebral disk is present between the atlas and epistropheus, as is found in ancestral forms.

The scapula is slightly retarded in its ossification, and the acromion is still cartilaginous. In the forelimb ossification is proceeding normally but the radius and ulna are twisted.

The development of the osseous skeleton has been studied from the 17-day-old



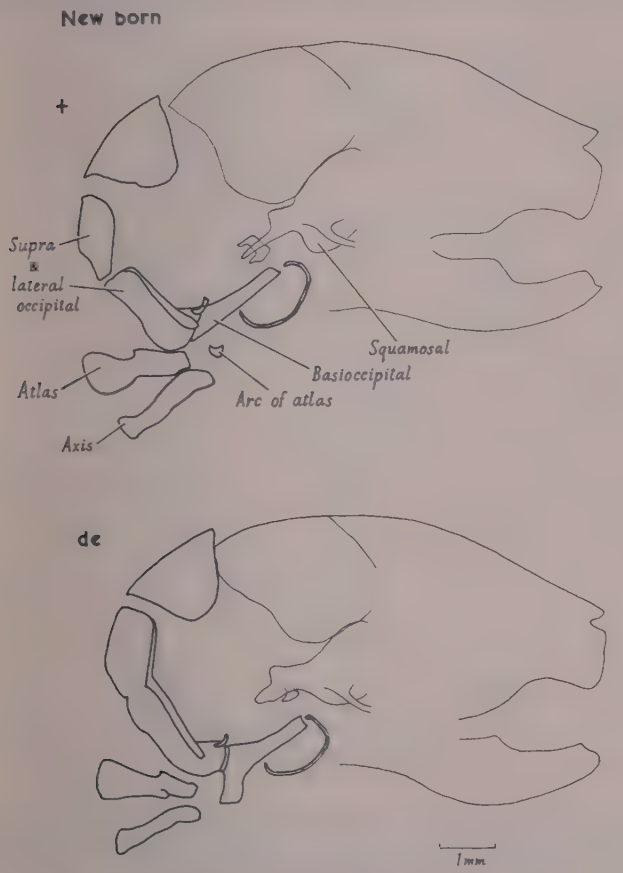
TEXT-FIG. 19. Development of the embryonic scapula and arm.



TEXT-FIG. 20. Ventral view of upper cervical region.

embryonic mouse to the 3-week-old post-natal mouse, by means of the alizarin clearance technique.

In the 17-day-old embryo several of the abnormalities seen in the adult are forecast. The glenoid process of the squamosum is wide. The clavicle is elongated, the scapula is displaced laterally, and the hole, if present, is only ossified



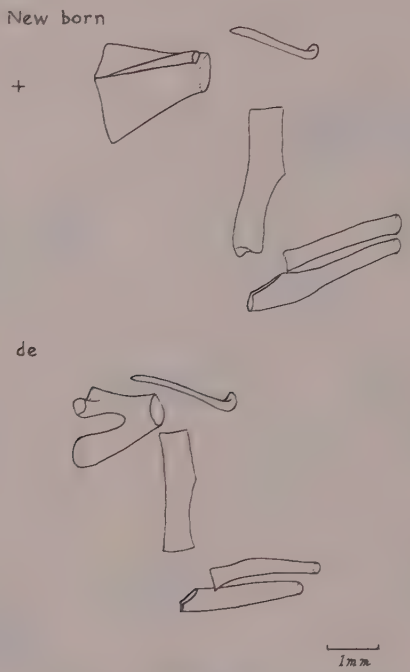
TEXT-FIG. 21. Lateral view of the skull and upper cervical vertebrae.

along part of its border, the dorsal border being cartilaginous. The deltoid ridge of the humerus is poorly developed, and the radius and ulna are twisted.

In the 18-day-old embryo the supra and lateral occipitals are already fused in droopy-ear, 12 days before their normal time. Normally the centra of the cervical vertebrae have now started to ossify, but in droopy-ear this is retarded and the dens shows no sign of any ossification at all.

In Text-fig. 21, a diagram of normal and droopy-eared skulls and anterior

cervical regions, four points which are quite typical at this stage of developmen should be noted. First, there is complete fusion of the supra and lateral occipitals Secondly, the form of the squamosum is indicative of the fusion to occur betwee the caudal and glenoidal processes. Thirdly, there is fusion of the basioccipita and the anterior arc of the atlas; this is rare at this age in droopy-eared mice, an might have resulted in the fusion of the atlas to the skull, as was once found in a



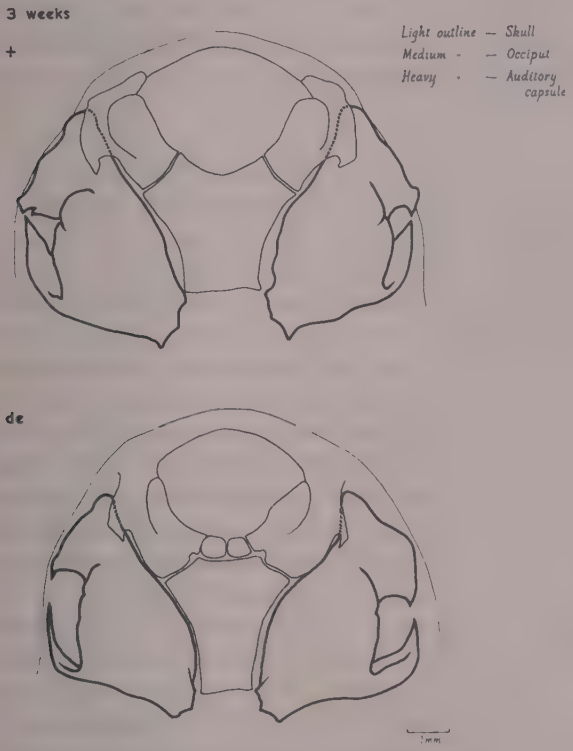
TEXT-FIG. 22. Lateral view of the clavicle, scapula, and arm.

adult mouse. The atlas and the axis are widely spaced and reduced in size Finally, although this is not illustrated in the diagram, the ossification of th centra of the first five vertebrae is retarded.

The abnormalities described in the 16-day-old embryo in the pectoral girdl and forelimb are still evident (Text-fig. 22). These bones were drawn while stil attached to the rest of the body in order to show their positions relative to on another. At 7 days old, post-natally, the interparietal is wide, the supraoccipita vertical, and the foramen magnum reduced in size. The dens, which started t ossify on the third day, 4 days later than normal, is now fused to the anterior ar of the atlas, instead of which it should have been connected to the axis since th thirteenth embryonic day. In the vertebral column the neural arches are foun to be late in fusing, especially in the cervical and thoracic regions.

At 14 days the neural arches of the atlas and the axis are still unfused, and the epiphyses of the forelimb are retarded, especially the trochlea of the humerus.

The diagram of the posterior region of the skull at 21 days (Text-fig. 23) shows the ventral position of the ear vesicles, and how this involves a narrowing of the basioccipital. The two ossified lumps on the occipital border of the basioccipital illustrate again the abnormality which occurs in this region. When fusion of the



TEXT-FIG. 23. Ventral view of the posterior region of the skull showing the pleuro-centrum at the posterior border of the basioccipital in *de*.

neural arches of the atlas and axis has occurred there is frequently an asymmetry of the two halves.

The developmental history of *de* has now been completed, and the abnormalities seen in the adult have been traced back to the 12-day embryo, where the first signs of the syndrome are delayed cartilage formation, and localized areas of disorganized cartilage which develop into the areas of specific abnormalities in the adult.

In view of the disorganized cartilage in the basioccipital and scapula, the internal bone structure was investigated to see if any abnormalities persisted in

the osseous skeleton. Whole bones were dissected, and histological sections of bones were examined of mice from birth to 175 days old. No striking abnormalities were found, but in all specimens the femur and humerus showed thin flat epiphyseal plates and a delay in the ossification of the epiphyses; the trabeculae were also retarded in development, and less regular in their pattern. In all, just over 40 mice were used for the dissection of bones, so the differences described above, though slight, are probably significant, and are likely to be dependent on the retardation of the earlier shapes in skeletal development. According to Chang (1949) the shape of the epiphyseal plate is closely linked to trabecular pattern, so the flattening of the epiphyseal plate (Text-fig. 12) is correlated with the disorganized trabeculae.

DISCUSSION

The majority of the skeletal abnormalities of the adult droopy-eared mouse have been traced either directly or indirectly to the condition in the embryo, where there is delay in ossification. This affects many parts, namely, the basioccipital, atlas, epistropheus, scapula, and all the long bones of the limbs. In most cases this has been traced back directly to delayed or abnormal chondrification, but in the case of the limb rudiments it has not been possible to detect differences in the time of onset of chondrification.

The cartilaginous abnormalities of the basioccipital, atlas, and epistropheus, so varied in their form, at first presented a problem. The condition in the 12-day embryo, however, was uniform; the mesenchymal condensation at the occipital border of the basioccipital was not rounded but blunt-ended, and projected ventrally (Plate 1, figs. A and C). At 14 days this projection forms a cartilaginous connexion between the basioccipital and the anterior arc of the atlas (Plate 1, figs. G and H) and the dens is split transversely into an anterior and posterior region. The whole of this situation is similar to that discussed by de Beer (1937) who points out that in mammals the hypocentrum of the pro-atlas is lost and the dens epistrophei is formed by a fusion of the pleurocentra of the pro-atlas and atlas, and it is both of these which fuse to the centrum of the epistropheus. Thus in droopy-ear the hypocentrum of the pro-atlas is not lost but retained in the cartilaginous stage, in the position which is normal for those members of the Amniota which retain this feature. The dens has divided into its component parts, with the centrum of the atlas in the atlas, and the centrum of the pro-atlas situated at the posterior border of the basioccipital (Text-fig. 23), a position similar to that found in birds, crocodiles, monotromes, and early therapsids; so that the situation in droopy-ear is similar to that found in earlier vertebrate forms. The cause of the variations found in droopy-ear is that the splitting of the dens into its component parts is not always complete and varying amounts of fusion are found.

The anomaly of the double thickening of the notochord within the dens in the 16-day embryo may also be explained by reference to those mammals which

retain the centrum of the pro-atlas at the base of the skull, in which case the centrum is pierced by the notochord (de Beer, 1937).

Although the anomalies of this region have one factor in common, in that they are illustrative of earlier vertebrate forms, this in itself does not explain their origin. However, it is of interest, from the point of view of evolution by gene mutation, to find that a mutation has provided a localized, but nevertheless clear, reversion to an earlier form.

Another anomaly of the basioccipital is that it is thin, and the cells are abnormal in their size and distribution; a very similar situation is found in the scapula. At a very early stage (13 days) in the scapula, chondrification frequently fails to take place in the centre of the posterior part of the blade, and there is a hole in this position in the bone. If chondrification failed to take place because of the thinness of the mesenchymal condensation, it is probable that the tegmen tympani, which is normally a thin sheet of bone, failed to be formed for a similar reason.

The abnormal fusion times may also be shown to be closely connected with the other abnormalities. Early fusion occurs between the supra and lateral occipitals. Both these bones suffer a ventrad shift in conjunction with the reduction in length of the sphenoid bones. It is easy to conceive that this shift could involve a slight change in the relative positioning of the two bones, and cause an abnormal fusion time. A similar change in fusion times in the occiput is seen in chondrodystrophic man (Rischbieth, 1912). Late fusion times are frequently found in the mouse mutants (*un*, *Sd*, *ch*) in connexion with retardation and reduction in size of ossification centres, as is easily understood. Thus the delay in fusions concerning the basioccipital, atlas, and epistropheus is explained. The reason for the late fusion of the frontals is probably connected with the increased width of the skull in this region. The late fusion of the ilium with the ischium and pubis has not been accounted for, but closer examination might well reveal retardation in this area.

Thus in general the abnormalities of the droopy-ear syndrome are due to an initial retardation apparent at the time of chondrification; and in the two areas most specifically affected by the droopy-ear gene, the anomalies have been traced back to abnormal chondrification and thin mesenchymal condensations. Further retrogressive analysis met with no success.

It is of interest to see droopy-ear in relation to other mouse mutants which have delayed chondrification. In undulated (*un*) the cartilage is histologically normal, but the acromion fails to chondrify owing to the thinness of the mesenchymal condensations. The atlas and epistropheus are abnormal, varying degrees of fusion being found between the two bones. The rest of the vertebral column and sternebrae may also be affected (Grüneberg, 1950). Congenital hydrocephalus has also been traced back to a stage of delayed chondrification. In this case the effect is more severe and the results are widespread throughout the skeleton (Grüneberg, 1953). In tail kinks the skeletal anomalies are found in the ribs,

sternum, and the vertebral column (Grüneberg, 1955). The syndrome of Danforth's short tail is attributed to an anomaly of the notochord, and skeletal abnormalities are found throughout the vertebral column. In the epistropheus the dens epistrophei is reduced and this has caused the atlas to have a single 'horse-shoe' shape articulation surface (Grüneberg, 1958). In each of these cases, although the mutants may have one or two similar areas affected, the form of the abnormality is different, and the other features of the syndromes are most diverse.

A closer comparison is provided by comparing the droopy-ear anomalies with those of some chondrodystrophic disorders as illustrated by creeper fowl (Landauer, 1931, 1932, 1933), dachs rabbits (Sawin & Crary, 1957), ancon sheep (Chang, 1949), and chondrodystrophic man (Knötzke, 1929; Rischbieth, 1912).

In all five disorders one of the areas of marked abnormality is the occiput. There is abnormal differentiation and retarded growth of the basioccipital, and the sphenoid bones are also retarded. Various anomalies of the condyles, and in the fusion times of the occipital bones, result in the foramen magnum being ventral. Another constant character is the shortening of limb-bones, although the relative amount of shortening differs in the various disorders, and is mildest in droopy-ear. But the comparison between the dachs rabbits and droopy-eared mice is worthy of special attention. In both cases the significant differences in the skull, atlas, and axis are extremely similar. The dachs rabbit also has the dens positioned in the atlas or at the base of the basioccipital.

Several theories have been put forward to explain why some parts of the skeleton should be more severely affected than others. Grüneberg (1955), writing on tail-kinks, explains regional abnormalities in terms of gene action of no definable 'period of activity', but suggests that in tail-kinks 'the effects become apparent when susceptible (small) sclerotomes pass through a critical phase of differentiation'. That a gene has no 'definable period of activity' with direct respect to time, but rather a period of activity primarily in respect to cell differentiation or chemical change, is the easier concept to understand. In many cases this may necessarily limit the activity of the gene to a certain period of time. In droopy-ear it would seem that the activity of the gene is associated with the early stage of mesenchymal condensations. At least this is when its effects first become apparent, but the gene may have affected the mesenchyme according to its regional differentiation which begins before the mesenchyme condenses.

The localization of specific abnormalities might also be dependent on the time of chondrification. Landauer (1931) noted that in creeper fowls the cartilage primordia which appeared earliest were the ones to suffer the greatest degree of abnormal differentiation. Certainly in the mouse the basioccipital, anterior arc of the atlas, and the scapulae are amongst the earliest to chondrify. Also, within any system where an allometric ratio between two parts exists, a general depression of growth rate or retardation will produce an effect which looks greater in the parts with the highest growth rate; this may be the case with the humerus, which has a higher growth rate than the radius and ulna.

There is a further theory that deals specifically with the failure of chondrification. It is that if the mesenchymal condensations fail to reach a critical size, then chondrification may fail to occur (Grüneberg, 1953). This is also upheld by droopy-ear, in that the mesenchymal condensations of the scapulae in the 12-day embryo are thinner than normal, and at 13 days there is no chondrification in the posterior part of the blade, and this part is not represented by a ligament in the adult mice. The problem now remains, why some mesenchymal condensations are thinner than others, and why in undulated mice the acromions are represented by ligaments, while the scapulae are whole, and in droopy-eared mice the scapulae are incomplete yet the acromions are partly ossified.

In conclusion it may be said that droopy-ear endorses the existing theories. The period of activity of the gene may be related to the time of formation of the mesenchymal condensations. Those which are formed earliest are the most affected, and as a result are thinner than normal, and chondrification may fail to occur.

All this tells us relatively little of what is basically happening in the droopy-ear syndrome, and the relationship of the skeletal anomalies with the change in distribution of the back and belly hair as seen on *a*^{t} and *A*^{y} backgrounds has not been investigated. But droopy-ear has proved itself to be an excellent mutant for developmental study and as a further example of a chondrodystrophic disorder it should be of great experimental value.

SUMMARY

1. Droopy-ear is a single, recessive, autosomal gene with full penetrance.

2. Droopy-ear is linked to Varitint *p*27 ± 3·2, but segregates independently of the other genes tested.

3. In adult droopy-eared mice the areas of specific abnormalities are the occiput and the shoulder girdle. The rest of the skeleton shows immaturity of form, and disproportionate shortening of the limb bones.

4. The anomalies of the adult skeleton are traced back, through a delay in ossification, to a delay in chondrification and localized areas of abnormal cartilage, and thus back to disturbed mesenchymal condensations, which in some cases are also thin. Certain of the abnormalities, in particular the splitting of the dens epistrophei into two parts, one of which may be attached to the basioccipital, strongly recall the anatomical situation in the more primitive vertebrates.

5. Some anomalies of the internal bone structure are described.

6. The droopy-ear syndrome is considered in relation to the syndromes of the other mouse mutants whose mesenchymal condensations are disturbed.

7. The similarities of the droopy-ear syndrome with some conditions in other chondrodystrophic disorders is noted.

8. The theories concerning the localization of specific abnormalities are discussed in relation to droopy-ear.

ACKNOWLEDGEMENTS

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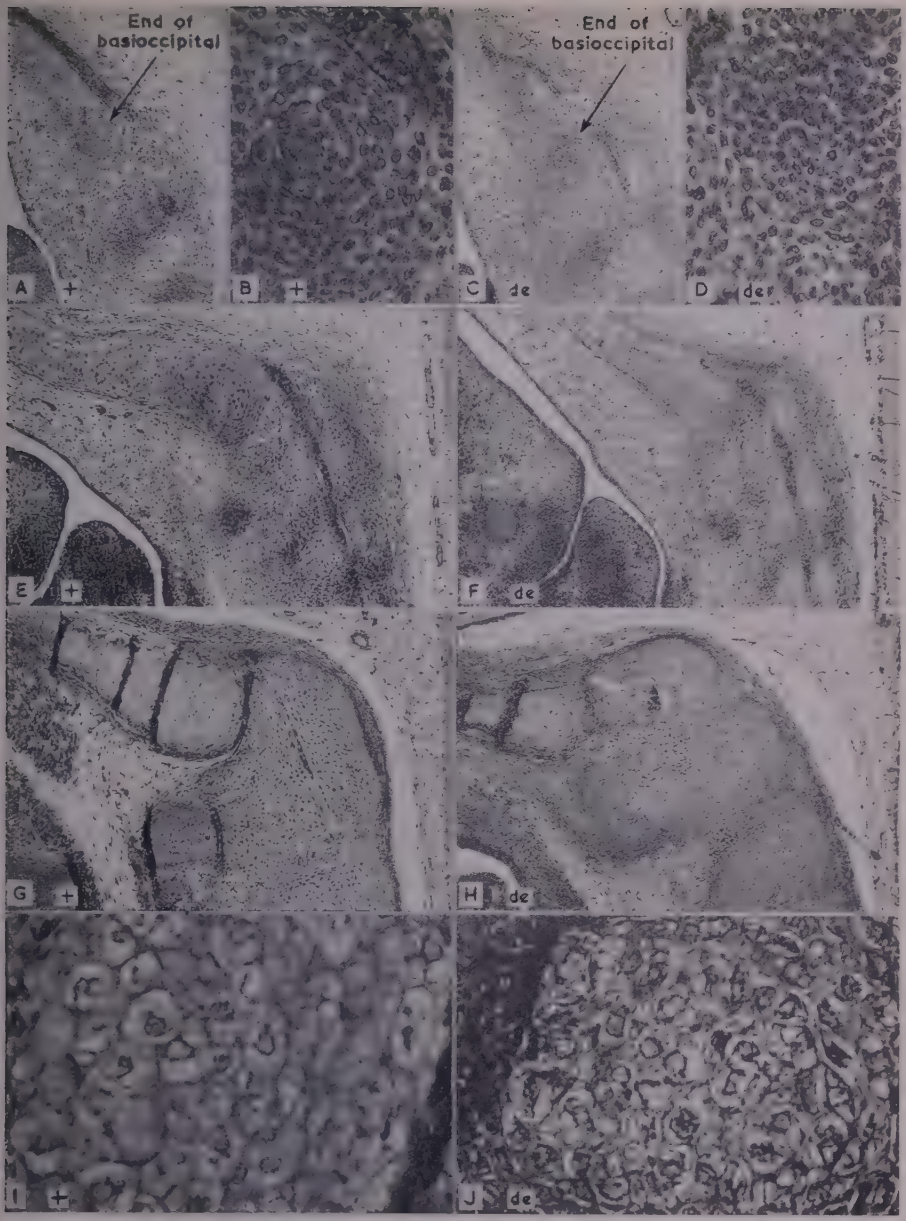
EXPLANATION OF PLATES

Plate 1

Figs. A and C. + and *de* 12-day embryos. Mid-sagittal sections of the basicranial cartilage and upper cervical vertebrae. ×90.

Figs. B and D. The posterior region of the basioccipital seen in Figs. A and C. ×800.



G. A. CURRY

Plate 1

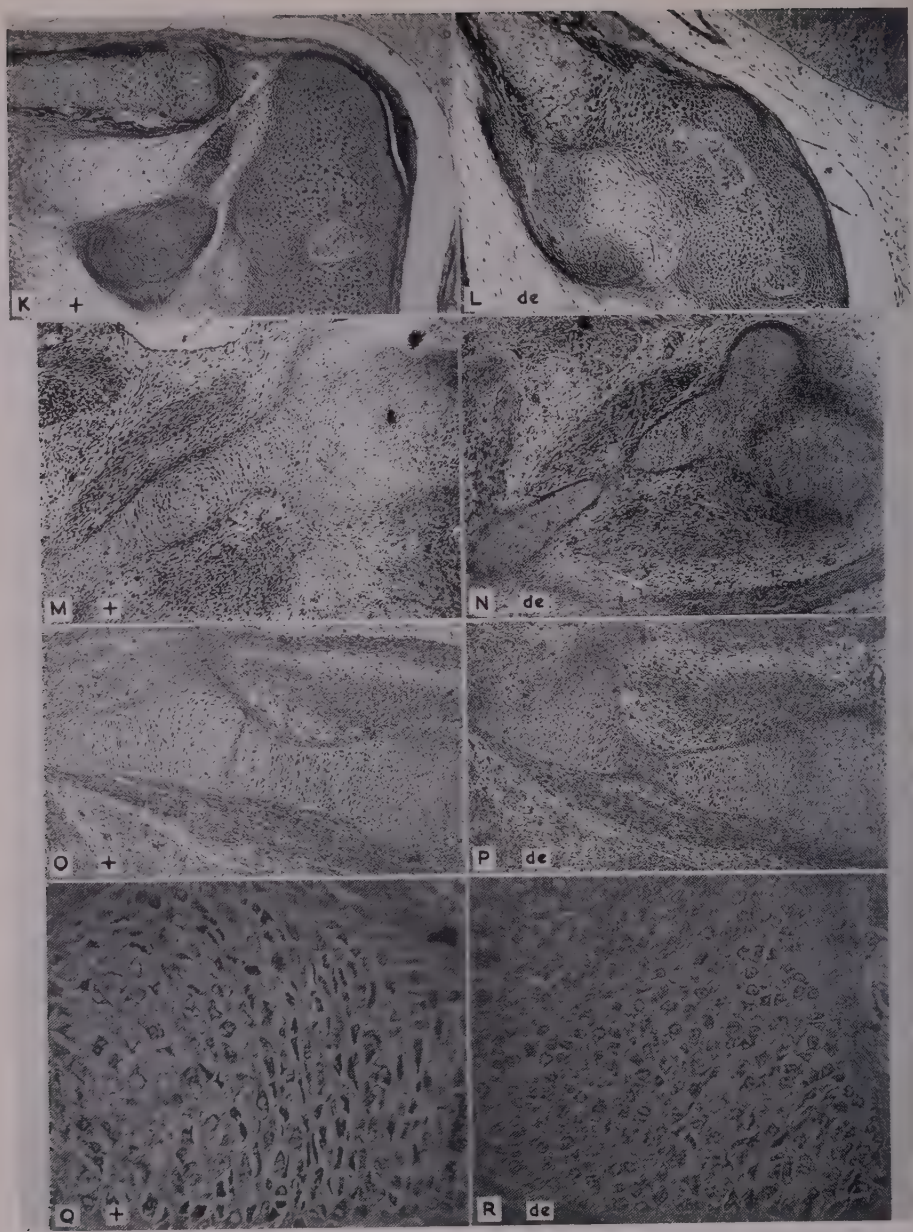


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Plate 2

FIGS. E and F. + and *de* 13-day embryos. Mid-sagittal sections of the basicranial cartilage and upper cervical vertebrae. ×90.

FIGS. G and H. + and *de* 14-day embryos. Mid-sagittal sections of the basicranial cartilage and upper cervical vertebrae. ×90.

FIGS. I and J. The posterior region of the basioccipital seen in Figs. G and H. ×800.

PLATE 2

FIGS. K and L. + and *de* 16-day embryos. Mid-sagittal sections of the basicranial cartilage and upper cervical vertebrae. ×90.

FIGS. M and N. + and *de* 13-day embryos. Longitudinal section of the scapula in the region of the base of the acromion. ×90.

FIGS. O and P. + and *de* 14-day embryos. Longitudinal section of the scapula in the region of the base of the acromion. ×90.

FIGS. Q and R. Base of the acromion seen in Figs. O and P. ×800.

(Manuscript received 16:vi:58)

Protein Accumulation in Early Chick Embryos Grown under Different Conditions of Explantation

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THE recent development of techniques originally devised by Waddington (1932) for the maintenance of the explanted chick embryo (Spratt, 1947; New, 1955; Wolff & Simon, 1955) has opened the possibility of determining quantitatively some parameters of the developmental processes occurring in embryonic tissues under these conditions. As a result of such measurements, protein accumulation in explanted embryos was found to be much smaller than in embryos developing in the egg. On the other hand, the progress of somite formation was found to take place at similar rates in embryos developing as explants or *in situ* (Herrmann & Schultz, 1958).

The slow rate of protein accumulation in the explanted embryos made it seem desirable to investigate whether under some other conditions of explantation protein accumulation would approach more closely the rate of protein formation observed in the naturally developing embryo. By varying the concentrations of the egg extract used as nutrient medium, and by enlarging the area of the extra-embryonic membranes explanted with the embryo, a considerable increase in protein formation could be achieved. The data bearing out these results are described in this paper.

MATERIALS AND METHODS

Cultivation of chick embryos in vitro. The cultivation method of Spratt (1947) was used in this study. Fertile eggs of 'Hyline' strain were incubated at 40–42 hours at 37½° C. to obtain 11–13 somite chick embryos. The culture chamber consisted of a Petri dish containing a thin cotton matting moistened to maintain proper humidity, and a watch-glass containing the agar gel (Fell & Robison, 1929).

Culture medium D (dilute egg constituents). This medium was that of Spratt (1947), with modifications introduced by Rothfels (1954). It was prepared by thoroughly mixing a whole egg (48–52 ml.) with an equal volume of physio-

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logical chick saline, which was composed of 0·7 g. of NaCl, 0·024 g. of $CaCl_2$, and 0·042 g. of KCl per 100 ml. of double distilled water. The above mixture was centrifuged at 1,800 r.p.m. at 3° C. for 1 hour. The extract, after being brought to room temperature, was mixed with an equal volume of saline-agar which was prepared by adding 1·5 g. of 'Difco' standardized agar to 100 ml. of chick saline. The saline-agar was liquefied by autoclaving for 15 minutes at 15 lb. pressure. No buffer systems were added because the extract was found to have sufficient buffer capacity. One-millilitre aliquots of this mixture (containing 25 per cent. yolk-albumen extract) were added to the watch-glasses and the culture plates were stored at 3° C. until used.

Culture medium C (concentrated egg constituents). This medium was prepared similarly to medium D except that the undiluted contents of a whole egg were mixed well and then added to an equal volume of saline-agar, giving a medium of twice the concentration (50 per cent.) of yolk-albumen extract as that of medium D.

Sterile procedures were followed in the preparation of culture plates and culture media and in the explantation technique.

Explantation procedure S (small extra-embryonic area). Fertile eggs incubated for approximately 40 hours were opened into an evaporating dish containing about 50 ml. of chick saline. A cut was then made into the vitelline membrane at the border of the blastoderm and continued along the entire circumference of the blastoderm. After removal of the blastoderm with the aid of forceps and a shallow 'spoon', the blastoderm was transferred to a Petri dish containing chick saline. The adhering vitelline membrane was removed with the aid of forceps and the embryo was examined under a dissecting microscope to determine the stage of development. The extra-embryonic area peripheral to the site of the sinus terminalis was trimmed off. The trimmed blastoderm was transferred in saline, with a large-bore pipette, to either medium D or C. The blastoderm (ventral side down) was flattened onto the agar gel and the excess saline removed. The culture plate was then incubated for the desired length of time at 37½° C.

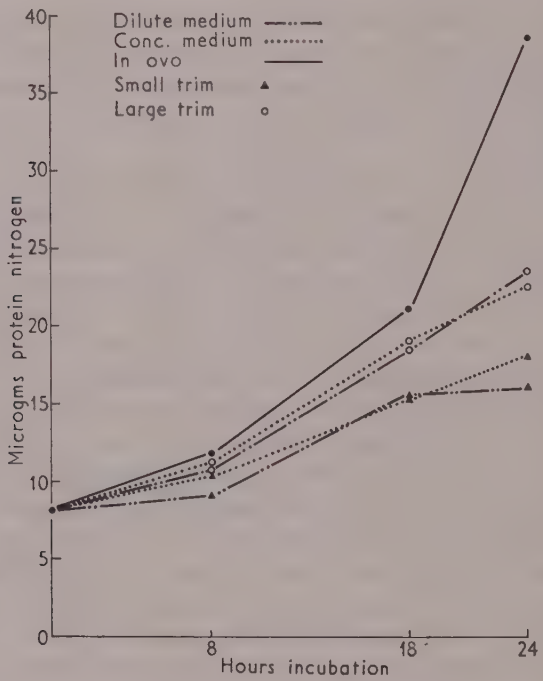
Explantation procedure L (large extra-embryonic area). Procedure S was first modified by altering the method of trimming the blastoderm prior to explantation. A circular cut was made around the blastoderm approximately 4 mm. peripheral to the sinus terminalis, including essentially the entire blastoderm. The explant was transferred in saline in a small 'spoon', floated from the spoon to the medium, and the excess saline removed as before.

After the desired incubation interval, the explants were removed from the culture plates with a large-bore pipette, transferred to chick saline, and trimmed by removal of the entire extra-embryonic portion of the blastoderm. Groups of 3 embryos were then placed in 2 ml. of cold trichloroacetic acid (5 per cent.). After thorough homogenization, the sample (3 embryos) was washed with three 2-ml. portions of cold trichloroacetic acid (5 per cent.) to remove amino-acids and small polypeptides. The nucleic acids were removed by washing with 2 ml.

of 5 per cent. trichloroacetic acid at 90° C. and the lipids were removed by extracting the homogenate with 2 ml. of 3:1 ethanol chloroform (70° C.). The protein nitrogen in the digest of the remaining residue was determined by Nesslerization.

RESULTS

Measurements of protein accumulation were carried out under four conditions of explantation: embryos with small extra-embryonic area on a medium of low concentration of egg constituents (S–D); embryos with small extra-embryonic



TEXT-FIG. 1. Protein nitrogen content of chick embryos developing as explants and *in ovo* at different periods of incubation. The embryos were explanted at the 11–13 somite stage after an incubation of about 40 hours (corresponding to the zero hour indicated in the graph). The protein nitrogen content is given per embryo.

area on a medium of high concentration (S–C); embryos with large extra-embryonic area on a medium of low concentration (L–D); embryos with large extra-embryonic area on a medium of high concentration (L–C).

For these four conditions, the protein contents of the embryos after explantation for 8, 18, and 24 hours is given graphically in Text-fig. 1 and numerically in Table 1. For comparison, the protein nitrogens are included for embryos grown

in the egg (controls). References to the statistical significance of the observed differences in the protein contents are indicated in Table 2.

TABLE 1

Protein nitrogen contents of chick embryos developing in ovo *and under different conditions of explantation*

The figures in the table represent protein nitrogen content (*PN*) in microgrammes per embryo at the end of the respective incubation periods; Δ is the difference between the protein nitrogen content at the end of two successively longer incubation periods (protein accumulation); 'per cent. of control' gives the protein accumulation as a percentage of that of the *in ovo* controls. The embryos were explanted at 11–13 somite stage (40 hours of incubation); their protein nitrogen content at this stage averages 8·2±0·36 microgrammes

Condition of cultivation	*8 hours' incubation*			*18 hours' incubation*			*24 hours' incubation*		
	PN	Δ	*Per cent. of control*	*PN*	Δ	*Per cent. of control*	*PN*	Δ	*Per cent. of control*
In ovo	11·8±0·41	3·6	..	21·1±0·75	9·3	..	38·4±1·51	17·3	..
S–D	9·0±0·14	0·8	22	15·4±0·28	6·4	69	16·0±0·33	0·6	3
S–C	10·3±0·17	2·1	58	15·2±0·32	4·9	53	18·0±0·44	2·8	16
L–D	10·7±0·22	2·5	70	18·4±0·51	7·7	83	23·3±0·97	4·9	28
L–C	11·2±0·24	3·0	83	19·0±0·73	7·8	84	22·5±0·73	3·5	20

TABLE 2

Statistical evaluation of the differences between the protein contents of the compared groups of embryo explants and of control embryos

The figures indicate the number of samples used in each group (*N*) and the *t* values for the differences between two groups of embryos. The differences which are significant on or below the 2 per cent. confidence level are indicated by *

Conditions of explantation		*Explantation period*					
		8 hours		*18 hours*		*24 hours*	
Extra-embryonic	*Egg*	*N*	*t*	*N*	*t*	*N*	*t*
Small (S)	Dilute (D)	49	6·53*	15	5·17*	30	7·09*
Large (L)	Dilute (D)	30		15		30	
Small (S)	Conc. (C)	30	3·00*	16	4·81*	31	5·29*
Large (L)	Conc. (C)	30		14		31	
Small (S)	Dilute (D)	49	5·91*	15	0·48	30	3·64*
Small (S)	Conc. (C)	30		16		31	
Large (L)	Dilute (D)	30	1·52	15	0·68	30	0·65
Large (L)	Conc. (C)	30		14		31	
Large (L)	Dilute (D)	30	2·34*	15	2·96*	30	8·44*
Control	..	35		20		18	
Large (L)	Conc. (C)	30	1·25	14	2·00	31	9·46*
Control	..	35		20		18	

Eight hours' explantation. During the first period of explantation the embryos of the S–D series show only an insignificantly small increase in their protein contents, whereas the protein contents of the S–C, L–D, and L–C embryos increase significantly. Actually, the protein content of the L–C series is not significantly lower than that of the controls after the initial explantation period. The values for both the L–D and S–C series are significantly lower than in the controls although they do not differ significantly from the value for the L–C series.

Eighteen hours' explantation. After this explantation period the protein content of the L–C series is still only insignificantly lower than that of the corresponding controls. The marked difference between the protein contents of the S–D and S–C series, observed after the 8-hour period, is no longer apparent, and the embryos in both S groups show significantly lower values than the embryos of both L groups.

Twenty-four hours' explantation. After the last period of explantation, the protein contents of the embryos in all explant series are found to be much lower than in the controls. This is due to the fact that the sharp increase in the protein content of the embryos developing *in vivo* does not occur in the explanted embryos. In the S–C, L–D, and L–C series the protein content increases at about the same rate as in the preceding explantation period, but in the S–D series practically no further increase in the protein content was found to occur.

These results show that embryos explanted with large extra-embryonic membranes show consistently the highest protein content at the end of each of the three explantation periods, as well as the highest rate of protein increase within each of these periods. In the L–C series, the protein content of the explants increases during the first 18 hours of explantation almost as fast as in the control embryos; the difference is not statistically significant.

In the embryos explanted with a large extra-embryonic area (L–D, L–C), no statistically significant difference in the measured or derived parameters could be found, irrespective of the extract concentration of egg constituents in the medium. This would indicate that under these conditions the effect of the nutrient concentration is negligible, and that the membrane size is the decisive factor for maximal growth of the explants. In explants with small extra-embryonic areas, a higher concentration of egg constituents in the medium causes a more marked increase in the protein content of the egg explants during the first and last periods of explantation.

DISCUSSION

The results of these experiments show that the increase of the protein content of explanted chick embryos depends upon the conditions of explantations, and can approach optimally the values found in embryos developing in the egg. In so far as differences in the protein content reflect corresponding differences in the rates of protein formation in the explants, the latter seems to be effectively con-

trolled by the explantation conditions used in these experiments. Such a control of protein formation provides a methodology which can be used to explore the relation of net protein accumulation and the uptake of labelled amino-acids into the protein moiety of the explanted chick embryo. The type of information which can be obtained by such an analysis is indicated in a paper by Loftfield & Eigner (1958). This approach also permits an investigation of the dependence of the accumulation of proteins, which are the end products of differentiation (e.g. cholinesterase in the nervous system), upon the rate of total protein formation, which is one of the indices of growth. Such an analysis may lead to a better understanding of the relation of differentiation to growth when both are defined in terms of protein formation.

Since both of the two tested conditions, viz. increased extra-embryonic membranes or increased concentration of egg constituents, lead to an increased protein formation in the explants, it could be proposed that either one leads to an improved nutrient supply from the medium to the embryo. A larger extra-embryonic area is of importance for the uptake of nutrients not only because of its larger contact surface for the absorption of nutrients from the medium, but also because it promotes establishment of a better extra-embryonic circulatory system in the explants. The complex nature of the utilization of yolk constituents by embryonic cells, recently discussed by Bellairs (1958), has to be considered in this context.

The use of a higher concentration of egg constituents is particularly effective during the first explantation period because it abolishes a marked lag period in the onset of protein accumulation. Whether this delay is related to transient suppression of proliferative activity in the explant is being investigated at the present time. To what extent the very marked retardation of the protein accumulation in the explants during the last period of explantation is due to an inadequately developed circulatory system, to some deficiency in the composition of the medium, or to some intrinsic condition of the explant itself has not been investigated so far.

Preliminary microscopic inspection of whole mounts of the explanted embryos shows that under optimal conditions of explantation, morphogenesis proceeds approximately as in the embryos developing in the egg. Under less favourable conditions of explantation, the morphogenesis of different organ rudiments may be affected to a different degree by the conditions of explantation. Under conditions of minimal growth, the brain structures seem to be less well developed than in the controls. On the other hand, the rate of somite formation is only little interfered with by different explantation conditions, as has been pointed out previously (Herrmann & Schultz, 1958). A more conclusive correlation of the progress of morphogenesis and explantation conditions presupposes the establishment of quantitative criteria for morphological differentiation of particular organ rudiments, which is beyond the scope of the present series of experiments.

SUMMARY

1. Chick embryos were explanted at the 11–13 somite stage to an agar medium and the protein content of the embryos was measured after 8, 18, and 24 hours of explantation.

2. An increase in the concentration of yolk-albumen in the medium as well as an increased extra-embryonic area was found to increase the protein contents of the explants.

3. In the presence of a large extra-embryonic area and a high concentration of yolk-albumen in the medium, the protein content of the explant was found to approach, during the first 18 hours of explantation, the protein content of the embryo developing in the egg.

ACKNOWLEDGEMENTS

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La Région caudale d'une Planaire est-elle capable d'induire la régénération d'un pharynx?

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INTRODUCTION ET POSITION DU PROBLÈME

Les expériences de Santos (1931), de Okada & Sugino (1937), et de P. Sengel (1951, 1953) ont mis en évidence les processus morphogénétiques qui président à la différenciation et à la régénération du pharynx des Planaires.

Santos (1931) greffe des fragments de tête à divers niveaux du corps de la Planaire *Planaria maculata*, et montre que le greffon céphalique induit la formation d'un pharynx supplémentaire dans les tissus de l'hôte.

Okada & Sugino (1937), par des expériences de greffes analogues à celles de Santos, démontre que la région prépharyngienne dépourvue de tête possède le même pouvoir inducteur que la tête.

A la suite de ces auteurs, P. Sengel (1953), reprenant les expériences d'implantation de fragments céphaliques chez la Planaire *Dugesia lugubris*, a pu montrer que la différenciation du pharynx supplémentaire induit se réalise en deux phases: le greffon céphalique induit d'abord, dans le corps de l'hôte, la différenciation d'une zone pharyngienne. Cette zone pharyngisée par l'induction céphalique, est capable, à son tour, de différencier un pharynx. Si l'on excise ce pharynx secondaire, en même temps que le greffon de tête, un nouveau pharynx se régénère dans la zone pharyngisée, en l'absence des tissus céphaliques qui avaient induit la zone pharyngienne supplémentaire.

L'ensemble de ces recherches révèle: (1) que les régions situées en avant du pharynx (région céphalique et prépharyngienne proprement dite) sont capables de transformer une région banale de la Planaire en une région pharyngienne; (2) que la région pharyngienne est capable de régénérer son pharynx en l'absence des régions antérieures.

Les régions prépharyngiennes (au sens large du mot) sont-elles les seules à posséder ce pouvoir inducteur d'une zone pharyngienne? R. Chandebois (1957 *a* et *b*), se fondant sur des expériences réalisées sur *Procecodes lobata*, pense que les régions post-pharyngiennes sont capables, au même titre que les régions prépharyngiennes, d'induire la différenciation d'un pharynx.

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De nouvelles expériences faites sur les Planaires *Dugesia lugubris* et *Polycelis nigra* nous permettent de répondre à la question posée et de trancher le problème.

PLAN DES EXPÉRIENCES

Nos observations et nos expériences ont porté sur des tronçons de Planaires, d'une part céphaliques prépharyngiens, d'autre part caudaux postpharyngiens. Les propriétés morphogénétiques des tronçons antérieur et postérieur d'une Planaire ont ainsi pu être étudiées isolément.

(1) Tout d'abord nous avons observé les processus de la régénération normale d'un tronçon céphalique et d'un tronçon caudal.

(2) Par des sections répétées, nous avons supprimé le blastème de régénération caudal, dans le cas du tronçon céphalique, et le blastème céphalique dans le cas du tronçon caudal, de manière à mettre en évidence les propriétés de chaque tronçon, en le soustrayant à l'influence du bourgeon de régénération.

(3) Nous avons empêché le bourgeon de régénération céphalique ou caudal de se former rapidement, en irradiant localement aux rayons X une région voisine de la section.

(4) Les expériences de sections répétées du bourgeon ont été refaites sur une autre espèce de planaire d'eau douce, *Polycelis nigra*.

Les résultats de ces expériences ont déjà été rapportés dans une note récente (Ét. Wolff, P. Sengel & C. Sengel, 1958).

MATÉRIEL ET TECHNIQUES

Les expériences ont porté sur les planaires d'eau douce *Dugesia lugubris* et *Polycelis nigra*.

Les fragments de planaires sur lesquels nous avons travaillé étaient placés 2 par 2 dans de petits cristallisoirs contenant 50 c.c. d'eau ordinaire, à 20°, et autant que possible à l'obscurité. Comme nous n'avons expérimenté que sur des fragments privés de pharynx, ils n'ont pas pu être nourris pendant toute la durée des expériences (1 ou 2 mois, selon les cas). La très grande diminution de taille qui en résulte n'est pas gênante pour l'observation, et n'empêche pas la régénération de se faire normalement.

Les techniques utilisées pour l'anesthésie, les amputations, les excisions et les irradiations aux rayons X ont été décrites par Ét. Wolff & F. Dubois (1947) et F. Dubois (1949).

Anesthésie: après un séjour de quelques minutes dans une solution de sulfate de nicotine à 1/10 000, les planaires sont parfaitement immobiles. Elles n'ont été anesthésiées en général que pour les expériences d'irradiation.

Amputations: les sections, qui doivent être franches et rapides, afin d'endommager le moins possible les tissus avoisinants, sont faites avec un éclat de lame de rasoir neuve fixée dans un porte-aiguille. La planaire est placée dans une

goutte d'eau sur un bloc de paraffine dont la surface horizontale est parfaitement lisse. Les opérations se font sous la loupe binoculaire.

Irradiations: les planaires sont d'abord anesthésiées, puis placées sur un tambour de soie où affleure de l'eau contenant 1 / 10 000 de sulfate de nicotine. Dans ces conditions, elles ne se dessèchent pas et restent immobiles aussi longtemps qu'elles se trouvent sur le tambour. La partie de l'animal qu'on ne veut pas exposer au rayonnement est protégée par un écran de plomb de 2 mm. d'épaisseur qu'on amène presque au contact de la planaire. Les planaires sont couchées sur le dos, de manière que la bouche et l'orifice génital, situés sur la face ventrale, puissent servir de repères. L'irradiation dure 20 minutes. Les animaux sont à 10 cm. de l'anticathode. Le courant est de 15 milliampères, sous une tension de 60 000 volts. Dans ces conditions la dose de rayons X est environ de 16 000 roentgen. Cette dose est suffisante pour tuer infailliblement tous les néoblastes de la région exposée au rayonnement (Ét. Wolff & F. Dubois, 1947).

RÉGÉNÉRATION NORMALE

Les planaires sont divisées en trois parties par deux sections transversales, dont la première se situe au niveau de la base du pharynx, et la deuxième juste en arrière de la bouche (fig. 1). On obtient de cette manière une région I,

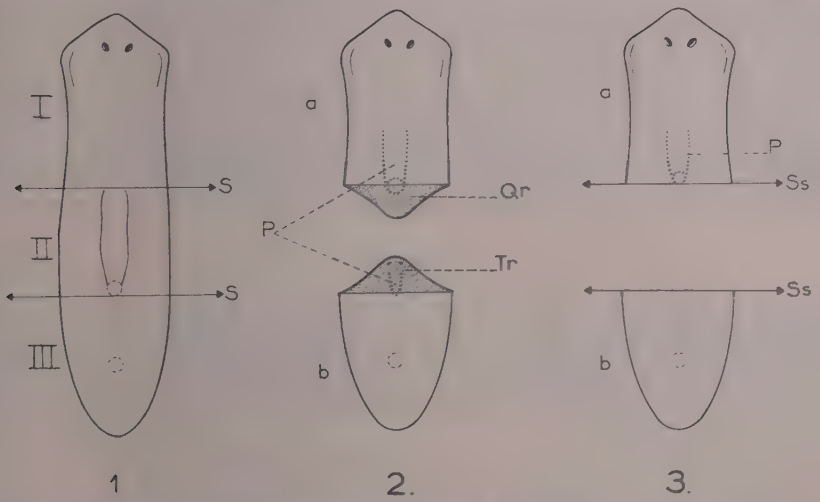

Fig. 1. Division de la planaire en trois tronçons I, II, et III. S, niveau des sections.

Fig. 2. Régénération normale de la région I (*a*) et de la région III (*b*). P, pharynx régénéré. Qr. queue en voie de régénération. Tr. tête en voie de régénération.

Fig. 3. Sections répétées du blastème de régénération caudal (*a*) et céphalique (*b*). P, pharynx régénéré. Ss, niveau des sections successives.

céphalique prépharyngienne, une région II, pharyngienne, et une région III, caudale post-pharyngienne. Les sections ont été faites avec le plus de précision

possible, de façon que jamais aucun morceau du pharynx ou de la zone pharyngienne ne reste attaché à la région I ou à la région III.

Nous avons suivi la régénération normale des régions céphalique I et caudale III.

(*a*) *Région I*

Deux à trois jours après la section apparaît un bourgeon de régénération caudal. Les jours suivants, en même temps que le régénérat grandit et prend la forme d'une queue, un pharynx se constitue dans les tissus anciens de la région I. La bouche apparaît à la limite entre les tissus anciens et les tissus régénérés (fig. 2*a*).

Dans ce cas, la régénération du pharynx est une morphallaxie, puisque c'est une zone autrefois prépharyngienne qui se transforme en zone pharyngienne. Le régénérat caudal, lui, est formé par épimorphose. Est-ce la tête ou le régénérat de queue qui induit la formation du pharynx par morphallaxie?

(*b*) *Région III*

Le tronçon caudal, isolé par la section passant en arrière de la bouche, édifie un blastème au bout de trois jours. Celui-ci reconstitue une tête, où les yeux, témoins de la présence du cerveau (Lender, 1950) et le pharynx se différencient en même temps: on les voit apparaître le dixième ou le 12^{e} jour. Ici aussi, la nouvelle bouche est située à la limite entre les tissus nouveaux et les tissus anciens de la queue (fig. 2*b*).

Mais le pharynx est formé par épimorphose dans le régénérat. Il semble bien que la nouvelle tête soit responsable de la formation du nouveau pharynx et de la zone pharyngienne. Toutefois on peut se demander si la queue seule ne serait pas capable de régénérer un pharynx.

SECTIONS RÉPÉTÉES DU BLASTÈME

Nous avons tenté de supprimer l'action que peut avoir sur les tissus anciens le bourgeon de régénération soit caudal (région I), soit céphalique (région III): pour cela il faut le sectionner chaque fois qu'il apparaît. Dans ces opérations, nous avons essayé de n'enlever que les tissus néoformés, en entamant le moins possible les parties anciennes.

(*a*) *Région I*

Le bourgeon caudal a pu être sectionné tous les 2 jours, ou même tous les jours dans certains cas. Au moment de l'amputation, il se présente sous forme d'une frange transparente de tissus néoformés, ne mesurant pas plus de 0,1 mm. dans le sens de la longueur de la planaire. Après trois à onze jours, on voit apparaître un pharynx dans la zone où il se forme habituellement au cours de la régénération normale (fig. 3*a*). La régénération du pharynx dans ces conditions a été observée dans 30 cas sur 45 (Tableaux 1 et 1 *bis*).

La zone où se trouve le pharynx régénéré est une nouvelle région pharyngienne formée par morphallaxie. Nous avons isolé cette région pharyngienne par une section transversale passant au niveau de la base du nouveau pharynx par une fente longitudinale pratiquée sur la face dorsale. Un nouveau pharynx apparaît au bout de quatre ou cinq jours, alors que ni la tête ni la queue ne se

TABLEAU 1

Tronçons I: sections répétées du blastème

Nombre de sections successives du bourgeon	*Date de l'apparition du pharynx (en jours)*	*Date de l'apparition du bourgeon caudal (en jours)*	*Nombre de pharynx régénérés*	*Nombre de planaires n'ayant pas régénéré de pharynx*
2	3–6	4–8	12	3
3	4–8	6–10	8	6
4	9–11	10–12	4	2
5	8–12	9–13	4	3
6	11	13	2	1
			Total: 30	Total: 15

sont encore différenciées. Nous sommes donc bien en présence d'une zone pharyngienne telle que l'a définie P. Sengel (1953), douée d'une certaine autonomie et capable à elle seule d'induire la différenciation du pharynx.

Lorsqu'on supprime le régénérat caudal chaque fois qu'il commence à se former, un pharynx se différencie malgré tout dans la région voisine de la section. Le régénérat n'est donc pas responsable des remaniements observés dans cette région, et ce n'est pas lui qui induit la régénération du pharynx par morphallaxie.

(*b*) *Région III*

Le bourgeon céphalique doit être enlevé chaque fois qu'il commence à s'édifier. Il n'a été coupé que tous les deux ou trois jours en moyenne, car il semble qu'il soit un peu plus lent à se reformer après chaque section que dans le cas de la région I.

Les expériences de sections répétées du régénérat céphalique ont porté sur plus de trois semaines, en ce qui concerne les tronçons caudaux III. Jamais nous n'avons observé la formation d'un pharynx en l'absence de tête (Tableau 2) (fig. 3*b*). Ensuite, dès que nous avons laissé celle-ci se différencier, le pharynx est apparu normalement dans le régénérat, en même temps que les yeux.

Il semble donc bien que la présence d'une région céphalique soit nécessaire à la régénération du pharynx: celle-ci est impossible à partir d'une queue seule.

TABLEAU 1 *bis*

Évolutions de 12 tronçons I

Dates (jours)	*Sections successives du bourgeon, apparitions du pharynx et du bourgeon, chez 12 individus*											
0	*Opération initiale*											
1												
2	1e section	1e section	1e section	1e section	1e section	1e section	1e section	1e section	1e section	1e section	1e section	1e section
3	2e section	2e section	2e section pharynx	2e section	2e section	2e section	2e section					
4	pharynx	pharynx	bourgeon			pharynx	pharynx	2e section	2e section	2e section	2e section	2e section
5	bourgeon			pharynx	pharynx							pharynx
6		bourgeon		bourgeon	bourgeon	bourgeon	bourgeon	pharynx	pharynx	pharynx	pharynx	
7										bourgeon	bourgeon	bourgeon
8								bourgeon	bourgeon			

TABLEAU 2

Tronçons III: sections répétées du blastème

Nombre de sections successives du bourgeon	*Durée des expériences (en jours)*	*Nombre de fragments*
8	24	3
7	16	3
6	16	12
5	11	2
4	11	10
3	7	13
		Total: 43

IRRADIATIONS

Le bourgeon de régénération a tendance à se former assez vite après les sections, et on peut se demander si, même très petit, il n'est pas capable d'exercer une action inductrice dans les parties anciennes avoisinantes. Pour remédier à cet inconvénient, nous avons fait appel aux rayons X. On sait (Ét. Wolff & F. Dubois, 1947) que ceux-ci ont la propriété de tuer toutes les cellules de régénération de la zone qu'ils traversent. Ils empêchent donc toute régénération, jusqu'à ce que des néoblastes venus d'une région non irradiée parviennent au niveau de la section. Il convient de rappeler ici que la migration des néoblastes a été mise en évidence grâce aux rayons X par F. Dubois (1949).

Nous avons irradié une bande de tissus voisine de la section et ayant toute la largeur du corps de la planaire. La longueur de la zone sera précisée dans chaque cas. Les néoblastes venus soit de la tête (région I), soit de la queue (région III), peuvent-ils reconstituer, en l'absence des blastèmes de régénération, un pharynx dans la zone irradiée, au cours de leur migration vers la section?

(*a*) *Région I*

Nous avons irradié les planaires laissées entières, en ne protégeant du rayonnement que la tête jusqu'en arrière des auricules, ou un peu plus loin (fig. 4*a*). Quelques heures après l'irradiation nous avons coupé les animaux au niveau de la base du pharynx. Les tronçons I ainsi obtenus présentent donc une bande irradiée longue de 1 à 3 dixièmes de la longueur totale de la planaire (fig. 4*b*). Une dizaine de jours après l'irradiation, des taches de nécrose apparaissent, principalement dans la région voisine de la section, et sur la ligne médiane ventrale. Souvent la nécrose s'accentue beaucoup au cours des jours qui suivent, et les tissus atteints se désagrègent spontanément, ce qui entraîne quelquefois la mort de la planaire. Dans d'autres cas, seule subsiste la partie qui n'a pas été irradiée. Par contre, lorsque la nécrose n'est pas trop grave (quand 1 10 ou 2 10 de la longueur de la planaire seulement ont été irradiés), la région irradiée guérit, et un pharynx se forme au voisinage de la section (fig. 4*c*). Quelques jours après, le blastème s'édifie à son tour, et la régénération se poursuit normalement

TABLEAU 3

Tronçons I: irradiations locales

Nombre de fragments irradiés	*Longueur irradiée*	*Dates de l'apparition*		*Pharynx apparus avant le bourgeon*	*Pharynx apparus le même jour que le bourgeon*	*Tissus nécrosés éliminés sans régénération de pharynx*	*Tissus irradiés guéris sans régénération de pharynx*	*Planaires mortes*
		du pharynx	*du bourgeon*					
28	$\frac{2}{10}$ à $\frac{3}{10}$	25–30 j.	30–35 j.	8	0	11	0	9 (après 15–20 j.)
26	$\frac{1}{10}$	15–26 j.	18–26 j.	19	5	2	0	0
Total: 54				Total: 27	Total: 5	Total: 13	Total: 0	Total: 9

TABLEAU 3 *bis*

Évolutions de 8 tronçons I irradiés

	Individus							
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*
Longueur irradiée	$\frac{2–3}{10}$	$\frac{2–3}{10}$	$\frac{2–3}{10}$	$\frac{2–3}{10}$	$\frac{1}{10}$	$\frac{1}{10}$	$\frac{1}{10}$	$\frac{1}{10}$
Mort	15 j.	18 j.						
Apparition du pharynx			27 j.		15 j.	15 j.	23 j.	26 j.
Élimination de tous les tissus irradiés				28 j.				
Apparition du bourgeon			31 j.	33 j.	18 j.	19 j.	24 j.	26 j.

(fig. 4*d*). Parfois, le nouveau pharynx et le bourgeon sont apparus le même jour (Tableaux 3 et 3 *bis*).

On peut déduire de cette expérience que les néoblastes migrateurs venus de la

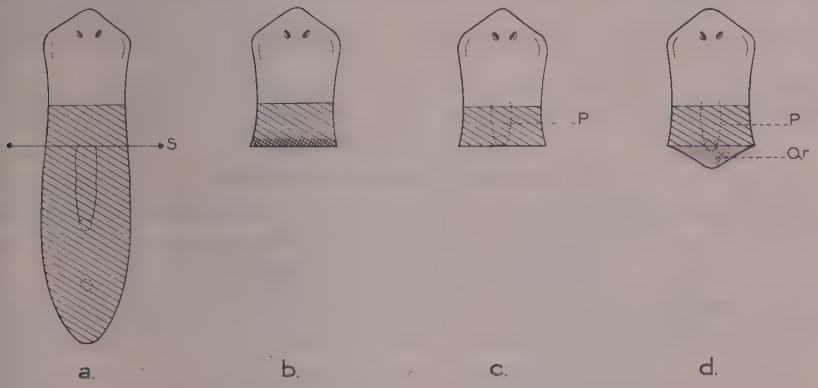

FIG. 4. Irradiation locale de la région I. (*a*) Schéma de l'intervention. (*b*), (*c*), et (*d*) Évolution du fragment après l'irradiation et la section. P, pharynx régénéré. Qr, queue en voie de régénération. S, niveau des sections. En hachures, zone irradiée. En quadrillé, zone de nécrose.

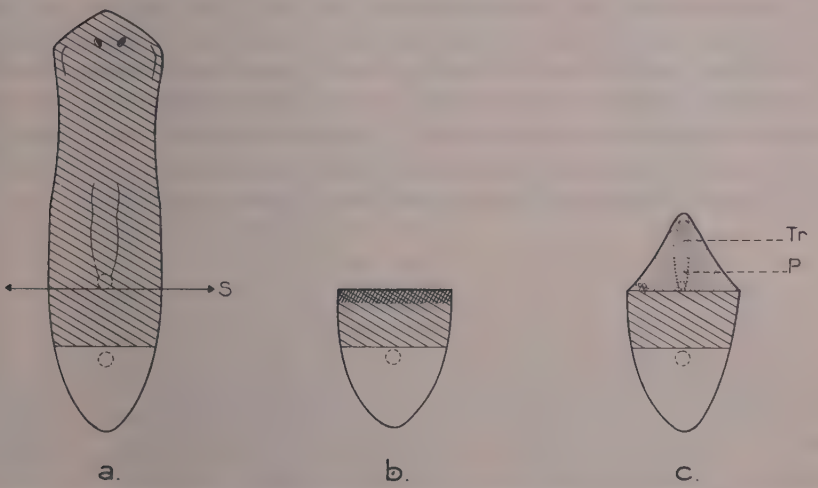

FIG. 5. Irradiation locale de la région III. (*a*) Schéma de l'intervention. (*b*) et (*c*) Régénération du fragment après l'irradiation et la section. P, pharynx régénéré. S, niveau des sections. Tr, tête en voie de régénération.

tête non irradiée forment un pharynx en passant au travers de la zone irradiée, avant d'édifier le bourgeon de régénération. En est-il de même pour des cellules de régénération migrant de la queue vers une région antérieure?

(*b*) *Région III*

Pour répondre à cette question, nous avons irradié localement la région III: toute la région antérieure de la planaire, jusqu'à l'orifice génital, est soumise à l'action des rayons X (fig. 5*a*). Puis on coupe la planaire transversalement en arrière de la bouche. Les tronçons ainsi formés présentent une région irradiée

TABLEAU 4

Tronçons III: irradiations locales

Nombre de fragments irradiés	*Longueur irradiée*	*Dates de l'apparition*		*Pharynx régénérés avant le bourgeon*	*Tissus irradiés éliminés (sans formation de pharynx)*	*Tissus irradiés guéris (sans formation de pharynx)*	*Planaires mortes*
		du bourgeon	*des yeux et du pharynx*				
20	$\frac{2}{10}$	20–30 j.	30–40 j.	0	5	11	4 (après 20–30 j.)

dont la longueur est égale aux 2 dixièmes de la longueur totale de l'animal (fig. 5*b*). Ils évoluent à peu près de la même manière que les fragments antérieurs: après 10 jours, nécrose près de la section, et sur une partie de la face ventrale. Parfois l'animal meurt, parfois tous les tissus irradiés et nécrosés sont éliminés. Mais en général la guérison se fait après un certain temps (une vingtaine de jours), et un bourgeon de régénération se constitue. Le même jour, des yeux et un pharynx y apparaissent, comme lors de la régénération normale (fig. 5*c*). Jamais un pharynx ne s'est formé avant le bourgeon de régénération (Tableau 4).

POLYCELIS NIGRA: SECTIONS RÉPÉTÉES DU BLASTÈME

Nous avons repris les expériences de sections répétées du blastème chez une autre espèce de planaire d'eau douce, *Polycelis nigra*. Nous avons obtenu les mêmes résultats qu'avec *Dugesia lugubris*: dans les tronçons I, le pharynx régénère par morphallaxie en l'absence de bourgeon caudal. Nous avons eu 16 cas positifs sur 25 planaires mises en expérience. Dans les tronçons III, au contraire, jamais nous n'avons vu un pharynx se former avant que le blastème céphalique ne se soit différencié en une tête pourvue d'yeux. Le pharynx régénéré se trouve dans les tissus néoformés, comme chez *Dugesia lugubris*.

Les résultats des expériences sur *Dugesia lugubris* sont donc valables également dans le cas de *Polycelis nigra*: dans les deux espèces, la morphogenèse du pharynx suit les mêmes lois.

CONCLUSION ET DISCUSSION

Les expériences de sections répétées du bourgeon de régénération montrent que les tronçons I sont capables d'induire la formation d'un pharynx, alors que les tronçons III ne le sont pas.

Si, en irradiant une région voisine de la section, on empêche le bourgeon de se former avant un temps relativement long, on observe les mêmes résultats: un pharynx se différencie en général dans un tronçon céphalique en l'absence de tout bourgeon, mais jamais dans un tronçon caudal.

On peut donc conclure que jamais un pharynx ne régénère à partir d'une queue seule, en l'absence de région céphalique, prépharyngienne ou pharyngienne. Par contre la tête seule peut induire la régénération d'un pharynx: dans ce phénomène le bourgeon de régénération caudal n'intervient pas.

Au cours de la régénération de la région I, c'est la tête, partie ancienne, et non le régénérat caudal, qui est l'inducteur de la transformation de la zone prépharyngienne en zone pharyngienne. Pendant la régénération du tronçon III, c'est au contraire le régénérat céphalique, et non la partie caudale ancienne, qui induit la différenciation du nouveau pharynx.

Contrairement à ce que pense Chandebois (1957 *a*, *b*) un régénérat antérieur céphalique possède donc un pouvoir inducteur dont le régénérat postérieur caudal est dépourvu. Cet auteur a obtenu, au cours de régénérations hétéromorphiques chez *Procerodes lobata*, la formation de queues avec pharynx en l'absence de tête, et à partir de fragments postérieurs; mais les régions prépharyngienne ou pharyngienne étaient conservées, en tout ou en partie. Ces résultats sont conformes à ceux de P. Sengel: le pharynx peut se régénérer si la région pharyngienne est conservée, à l'exclusion de toutes les parties antérieures au pharynx. Chandebois ne cite d'ailleurs aucun cas de régénération du pharynx à partir d'une région postpharyngienne seule.

D'autre part, Chandebois a observé que, dans le cas où la section se contracte en V, le pharynx ne se différencie pas, même en présence de la tête. Si le pharynx ne se forme pas, c'est que dans ces conditions la régénération devient très difficile: le blastème apparaît fort tard, et il se présente assez anormalement. De toutes manières, ce cas spécial ne permet pas de conclure que la tête n'a pas le pouvoir d'induire la régénération du pharynx. En effet, dans nos expériences, la très grande majorité des fragments I régénèrent tout à fait normalement.

RÉSUMÉ

1. Des expériences d'amputations et d'irradiations locales ont été faites sur les planaires d'eau douce *Dugesia lugubris* et *Polycelis nigra*, afin de préciser les rôles de la tête et de la queue dans l'induction d'une zone pharyngienne et d'un pharynx.

2. Au cours de la régénération normale d'un tronçon céphalique prépharyn-

gien (I), un pharynx se différencie dans les tissus anciens du fragment, en même temps que s'édifie le bourgeon de régénération caudal.

Au cours de la régénération normale d'un tronçon caudal postpharyngien (III) le pharynx apparaît dans les tissus régénérés, en arrière de la nouvelle tête. Celle-ci est alors déjà pourvue d'yeux.

3. Le bourgeon de régénération caudal des tronçons I a été sectionné tous les jours ou tous les deux jours. Un pharynx se différencie au bout de quelques jours dans le fragment au voisinage de la section.

Parallèlement, le bourgeon céphalique des tronçons III a été supprimé tous les deux jours. Aucun pharynx n'a pu régénérer dans ces conditions.

4. Les rayons X appliqués localement à une zone voisine de la section empêchent la régénération des tronçons I et III pendant un temps relativement long.

Un pharynx se différencie dans les fragments I bien avant que le bourgeon de régénération n'apparaisse.

Dans les tronçons III, il ne se forme jamais de pharynx avant que le bourgeon de régénération ne se soit différencié en une tête pourvue d'yeux.

5. Les résultats des expériences sont valables pour les deux espèces *Polycelis nigra* et *Dugesia lugubris*.

6. La régénération du pharynx est donc possible à partir d'une région céphalique prépharyngienne, en l'absence de tout blastème.

Elle est impossible à partir d'une queue seule en l'absence de régions céphalique, prépharyngienne ou pharyngienne.

SUMMARY

1. Experiments involving amputation and irradiation have been made on the fresh-water planarians *Dugesia lugubris* and *Polycelis nigra* to ascertain the precise roles of head and tail (Fig. 1) in the induction of a pharyngeal zone and of a pharynx.

2. During the normal regeneration of a prepharyngeal cephalic fragment (region I, Fig. 2*a*), a pharynx regenerates in the original tissues of the fragment at the same time as the caudal blastema appears. During the normal regeneration of a postpharyngeal caudal fragment (region III, Fig. 2*b*), the pharynx appears in the new tissues of the blastema. The eyes of the regenerated head have by then already appeared.

3. The caudal blastemata of cephalic fragments were cut away every day or every second day. A pharynx appears after a few days in the fragment (Fig. 3*a*). The cephalic blastemata of caudal fragments were cut away every second day. No pharynx regenerated under these conditions (Fig. 3*b*).

4. A zone close to the cut was irradiated with X-rays, which prevents the fragments regenerating for a long time. A pharynx appears in the cephalic fragment long before the regeneration blastema. In the caudal fragments, the

pharynx cannot regnerate before the blastema has formed. It appears only after the blastema has differentiated into a head (Fig. 5).

5. The same results have been obtained in another species of planarian, *Polycelis nigra*.

6. The regeneration of the pharynx is therefore possible in a cephalic fragment, devoid of any blastema. The regeneration of a pharynx is impossible in a tail without cephalic, prepharyngeal or pharyngeal regions.

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Studies on the Mode of Outgrowth of the Amphibian Pronephric Duct

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WITH ONE PLATE

INTRODUCTION

ALTHOUGH the manner in which the developing amphibian pronephric duct extends posteriorly has been studied rather extensively, it has not been clear whether this posterior outgrowth is largely the result of proliferative activity or whether it is due to migration of cells from an anterior level (Cambar, 1949). Holtfreter (1939) has demonstrated a marked migratory tendency in these cells from early gastrula stages in *Rana esculenta*, but a high mitotic count towards the posterior duct tip has been reported in *Triton alpestris* (Mollier, 1890), suggesting that proliferation may play a significant role. Furthermore, proliferation appears to be an important factor in elongation of the pronephric duct of the chick (Overton, 1958). This problem has now been examined more closely in a number of amphibian species in the hope that a more precise understanding of the mode of duct outgrowth might throw some light on the question of how the direction taken by the outgrowing duct is controlled. In experiments with anuran and urodele embryos (for reviews, see Holtfreter, 1944; Cambar, 1949; Fraser, 1950; Burns, 1955) there is evidence that the normal duct path exerts some influence on the direction in which the duct extends. Conceivably such an influence could be exerted in part through enhancement of terminal proliferation; however, counts of resting and dividing cells throughout the duct in various developmental stages, together with the behaviour of explants grown in plasma clots, strongly suggest that duct outgrowth in the species studied is an essentially migratory phenomenon.

MATERIALS AND METHODS

Experimental material consisted of *Ambystoma opacum*, *A. punctatum*, and *A. tigrinum* embryos, *Ambystoma* species collected locally (probably *A. punctatum* and *A. jeffersonianum*), and *R. pipiens* embryos obtained by the method of Rugh (1934). Embryos were kept on a water table where the temperature

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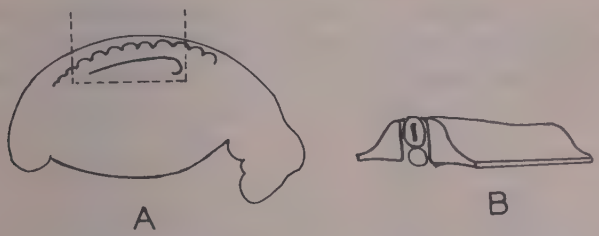
range was obtained by recording the temperature three to four times during a 24-hour period.

For mitotic studies urodele embryos were fixed in Bouin's at Harrison stages 29 to 34 (Rugh, 1948), stage 34 being the time at which the duct reaches the cloaca. Embryos were cut in cross-section and stained in Harris' haematoxylin. Mitotic figures in late prophase through telophase were counted in every section of the duct, those occurring in more than one section being recorded only once. The number of resting nuclei in each section was also counted. The relative frequency of mitotic figures was obtained for each successive group of ten sections, or 100 μ lengths of the duct, and expressed as mitoses/1,000 nuclei. This index, although adequate as a basis for comparing mitotic activity in one part of the duct with that in another, is, of course, not an accurate measure of mitotic frequency (see Abercrombie, 1946). Since ducts were of different lengths in different embryos, or even on right and left sides of the same embryo, in averaging counts



TEXT-FIG. 1. A, location of explanted region. B, explanted region spread flat with ectoderm removed.

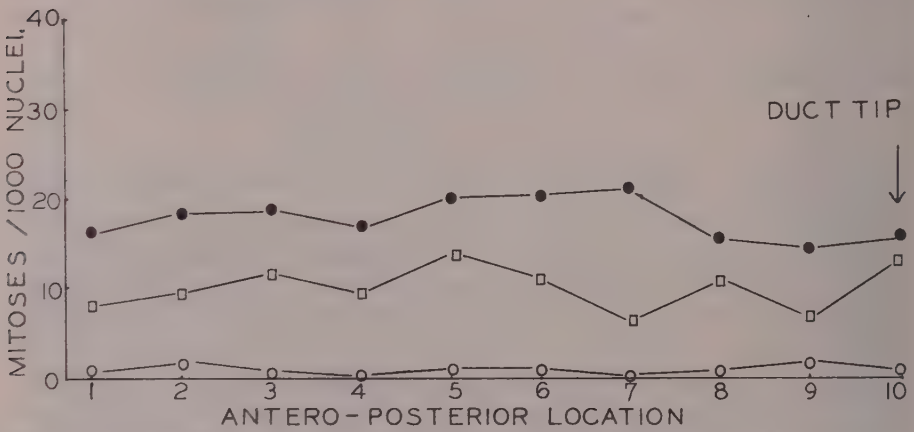
from different cases the posterior end of the duct was taken as the point of reference. The posterior tip of the duct could be clearly distinguished in *A. tigrinum* embryos, but this was not always true in *A. opacum* and *A. punctatum* embryos. In such cases several sections beyond the point where the duct could be clearly identified were included in the examination. Mitotic counts were made in epidermis of these same embryos for purposes of comparison. Cell counts were made in epidermis of one section of the trunk, and mitoses were counted in five alternate sections. Mitotic indexes arrived at on this basis are not comparable with indexes as determined for the pronephric duct for purposes of assessing relative mitotic frequency, but are nevertheless useful in comparing trends towards greater or less activity in different developmental stages.

In experiments with explants embryos of *R. pipiens* were used as well as those of the three urodele species. In preliminary experiments the nephric ridge was isolated from embryos in Harrison stages 23 to 28, or in equivalent stages in frog embryos, and cultured in a variety of media. In a few cases *R. pipiens* explants cultured in Holtfreter's solution on glass developed sprout-like extensions, although such explants generally developed very poorly. Therefore another method was devised in which a sizable portion of the embryo was explanted (see

Text-fig. 1). A saddle of mesoderm, including the notochord and neural tube, was removed and stripped of ectoderm. The posterior edge of the explant was usually cut to include, but in some cases to exclude, the posterior duct tip. Such explants were cultured first in a variety of media, and finally in a soft clot consisting of Holtfreter's solution (modified by the addition of dextrose, 1 part per thousand, and 0·5 per cent. sodium sulphadiazine), amphibian peritoneal fluid (see Rugh, 1948, p. 228), and freshly drawn chick plasma, 1:1:1. Chick embryo extract suitably diluted was later substituted for peritoneal fluid, and used with commercially prepared desiccated chick plasma, giving essentially the same results, although the first medium appeared somewhat more satisfactory. Explants were cultured on coverslips in depression slides. Cultures were maintained at room temperature (20–23° C.) except for a single series cultured at 27° C.

RESULTS

Spatial mitotic distribution. Counts of resting and dividing cells were made throughout the pronephric duct in three urodele species. These counts included 29 cases in *A. opacum*, stages 29–34 (14–15° C.); 23 cases in *A. tigrinum*, stages 29–33 (16–18° C.); and six cases in *A. punctatum*, stage 30 (18° C.). Although

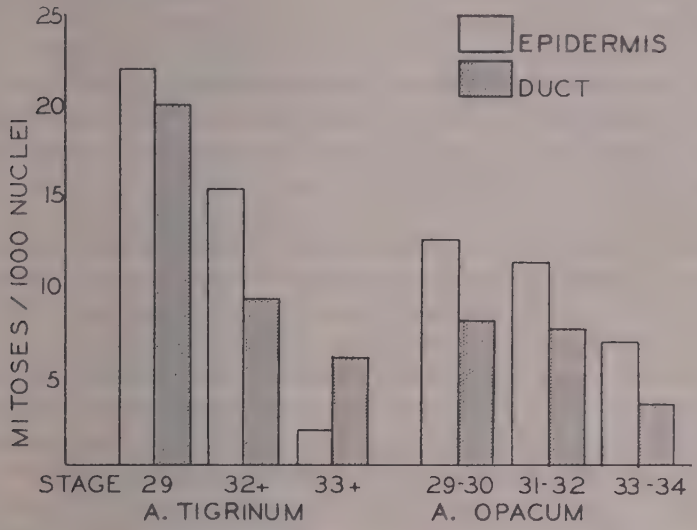


Text-fig. 2. Spatial distribution of mitotic activity in the elongating pronephric duct. Solid circles, *A. tigrinum*; open circles, *A. punctatum*; open squares, *A. opacum*. For explanation see text.

there were differences in the mitotic frequency between embryos of different species, and between different cases within the same species, there appeared to be no trend in individual cases towards anything other than a random spatial distribution of mitotic activity. Since the cell count in any single 100-μ length of the duct is small, it seemed possible that combined counts would show some trend. However, as illustrated in Text-fig. 2, combined values for the three species indicate no tendency towards high terminal proliferation. On the contrary, the

mitotic frequency is fairly regular throughout the length of the duct. Differences in overall mitotic activity which occur presumably reflect differences in growth rates of the three species.

Temporal mitotic distribution. If earlier and later stages are compared within the same species, although no spatial pattern in mitotic activity is evident, there is a definite trend towards lower mitotic frequency in later stages. Counts in *A. tigrinum* and *A. opacum* for entire pronephric ducts are grouped by stages and compared in Text-fig. 3.



TEXT-FIG. 3. Temporal mitotic pattern in the developing pronephric duct compared with that in epidermis. See text.

In the two species studied, it can be seen that the mitotic index is high in earlier stages, while the duct is growing posteriorly towards the cloaca. It drops gradually to a lower level as the duct reaches the cloaca at about stage 34. This drop, however, probably reflects only a general decrease in growth rate of the embryo, since the mitotic frequency in epidermis in these same embryos shows a similar trend (see Text-fig. 3).

In evaluating the role of proliferation in outgrowth of the duct, although no terminal proliferative region is present, the possibility must be considered that the entire duct material grows at a rather higher rate than the rest of the embryo during this period at which it is extending. Although Text-fig. 3 would suggest that there is no very marked difference between pronephric duct and epidermis, as was indicated above, the duct cell count was subject to a counting error while the mitotic count was not. Since nuclear size is large (*c.* 10 μ) compared to section thickness, this makes the actual difference in mitotic frequency between epidermis and duct considerable (see Abercrombie, 1946). Duct cells, then, have a

higher mitotic index than epidermis, but a comparison with epidermis alone is incomplete and provides very little basis for evaluating the role of proliferation. It must also be remembered that the mitotic index, however accurate, is not itself a measure of proliferation. Although it is clear that proliferation occurs during duct outgrowth, there is no good evidence from mitotic data presented here that proliferation is a critical factor in permitting elongation of the duct. On the contrary, the observation that in a dissection, in surface view, the elongating strand of pronephric duct material appears to decrease in diameter by almost one-half between stages 29 and 34, suggests that elongation occurs largely through shifting of cells rather than proliferation.

Migratory propensity. If an explant such as that pictured in Text-fig. 1 is placed in a suitable medium, one might expect outwandering of cells on all sides. However, if the migratory propensity of pronephric duct cells is particularly high one would expect first to find the elongating duct cords extending into the medium from the posterior edge of the explant. This expectation was confirmed when explants were placed in plasma clots, though not usually in the diagrammatic manner illustrated in fig. A of the Plate. Here the two outgrowths extend posteriorly from the same place and in the same direction as they would have in the normal embryo. These extensions tend to be cylindrical in shape and to have a diameter roughly similar to that of the normal developing duct (see fig. B of the Plate). In two cases in which such extensions were sectioned, they appeared to be roughly spherical in cross-section, and to adhere closely to the clot. No particular orientation of cells or polarity is evident within such an outgrowth. This is also the case in very early developmental stages of duct elongation in the embryo. Conceivably, sprouting of blood-vessels into the clot might have occurred also but there was no indication from the character of the outgrowing cells or their arrangement either in living cultures or in sections that this was the case. When explants are grown on the glass surface beneath a clot, the same apparently pure epithelial outgrowth occurs, but it takes a different form. Cells spread out in a sheet, though a tendency towards linear outgrowth is still evident as indicated by occasional tongues of cells which move out from the advancing edge (see fig. C of the Plate). Cylindrical cords may extend into the clot in greater or fewer numbers, and in almost any direction relative to the anteroposterior orientation of the explant, as illustrated in figs. D, E, F, and G of the Plate. The direction of outgrowth from twenty explants (a total of 61 outgrowths) was traced from photographic negatives. These tracings were superimposed and no definite preferential direction of outgrowth was indicated. It seems probable that rather small differences in operative technique determine the particular pattern of the outgrowth in so far as the origin of these extensions is concerned. Cylindrical extensions occurred whether the posterior tip of the duct was included in the explant or not, and so it seems likely that a small cut or tear at the edge of the explant anywhere might permit outgrowth of duct cells into the clot. This would allow outgrowths at anterior levels which sometimes occur. Once

outgrowth begins, it does not always continue in the same direction, that is, these extensions may bend (see Plate).

The first outgrowth from explants occurred after 1 to 2 days and these cords usually reached their fullest extent within a 24-hour period. Outwandering of other cell types occurred only later, making the originally smooth external contour of the explant highly irregular (compare figs. D and H of the Plate). In some cases the original epithelial cord became secondarily covered by fibroblasts or chromatophores which migrated out over its surface, and occasionally the nephric outgrowth became entirely concealed by abundant outwandering of all cell types. Such explants, though extremely diffuse, retained the main elements of their original organization. They remained in a healthy condition for as long as 2 weeks and showed indications of neuro-muscular differentiation as evidenced by sporadic twitching. Nephric rudiments developing within the explant became tubular, and often distended. Most cultures were maintained for only a week or less, since by this time all the changes described above had occurred. In one series of cultures kept at 27° C., there was rapid abundant outgrowth from the explant in all directions, so this procedure was abandoned.

Nephric outgrowths were obtained from explants of all three urodele species studied as well as from *R. pipiens*. Although mitoses occurred in these cultures, they did not appear to be particularly related to outgrowth of nephric cords. When two or more explants, or explants and cloacal rudiments, were placed in the same clot at varying distances and at various angles with respect to one another, no indications were obtained of any specific attractive effects such as occur *in vivo* (see, for example, Holtfreter, 1944; Bijtel, 1948).

DISCUSSION

Any assessment of the role of proliferative activity in duct elongation must, of course, be made on a comparative basis and considered in connexion with the geometric nature of the outgrowth. Although the data presented here are in some respects incomplete, as mentioned above, they are still sufficiently extensive to indicate that proliferation is probably unimportant as a factor in elongation of the duct. There is no indication of terminal proliferation, nor of any high proliferative rate during the period of duct outgrowth which does more than reflect the generally high growth rate of the embryo as a whole.

The migratory propensity of duct cells, although marked, is not unusually great. Migration of chromatophores, for example, appears to be equally rapid and extensive. However, at the developmental period coinciding with posterior extension of the duct, these epithelial cells have a higher migratory propensity than other cell types. Such a propensity has been demonstrated as early as the gastrula stage in *R. esculenta* (Holtfreter, 1939). Results of experiments in which the posterior part of the duct was excluded from the explant confirm the opinion of Nieuwkoop (1947) that 'Every single cell of the Wolffian duct rudiment must

have the power to migrate caudalwards. . . .' Duct elongation thus appears to be an essentially migratory phenomenon.

A tendency towards linear outgrowth of nephric epithelial cells was described by Holtfreter (1939), who observed pure epithelial cells extend in a band from explanted nephric material cultured on glass. Results of the present experiments confirm this observation and also suggest that not only the linear nature of the outgrowth but to some extent the cylindrical form and approximate calibre of the nephric cords can develop independently of a cellular environment.

Typically, epithelium cultured alone grows in sheets, and forms tubules only when embedded in fibroblasts, a phenomenon which has been extensively analysed recently (Grobstein, 1954). Although the outgrowths described here never develop a tubular structure, the ability of the plasma clot to support the development of some aspects of normal morphology is reminiscent of the report of Chlopin (1930) that tubules of the pancreas would form in a plasma clot in the absence of fibroblasts.

SUMMARY

1. The mode of outgrowth of the pronephric duct has been investigated by examining the mitotic index and migratory propensities of duct cells.

2. There is no indication of duct elongation through terminal proliferation or of high proliferative activity throughout the duct that does not merely reflect the generally high growth rate of the embryo as a whole.

3. Explants of the trunk region cultured in plasma clots showed cylindrical extensions of pronephric duct cords into the clot prior to outgrowth of other cell types.

4. It is concluded that in the species studied, duct outgrowth is an essentially migratory phenomenon, and that the nephric epithelial cells have the ability to form cords of roughly normal diameter in a non-cellular environment.

ACKNOWLEDGEMENT

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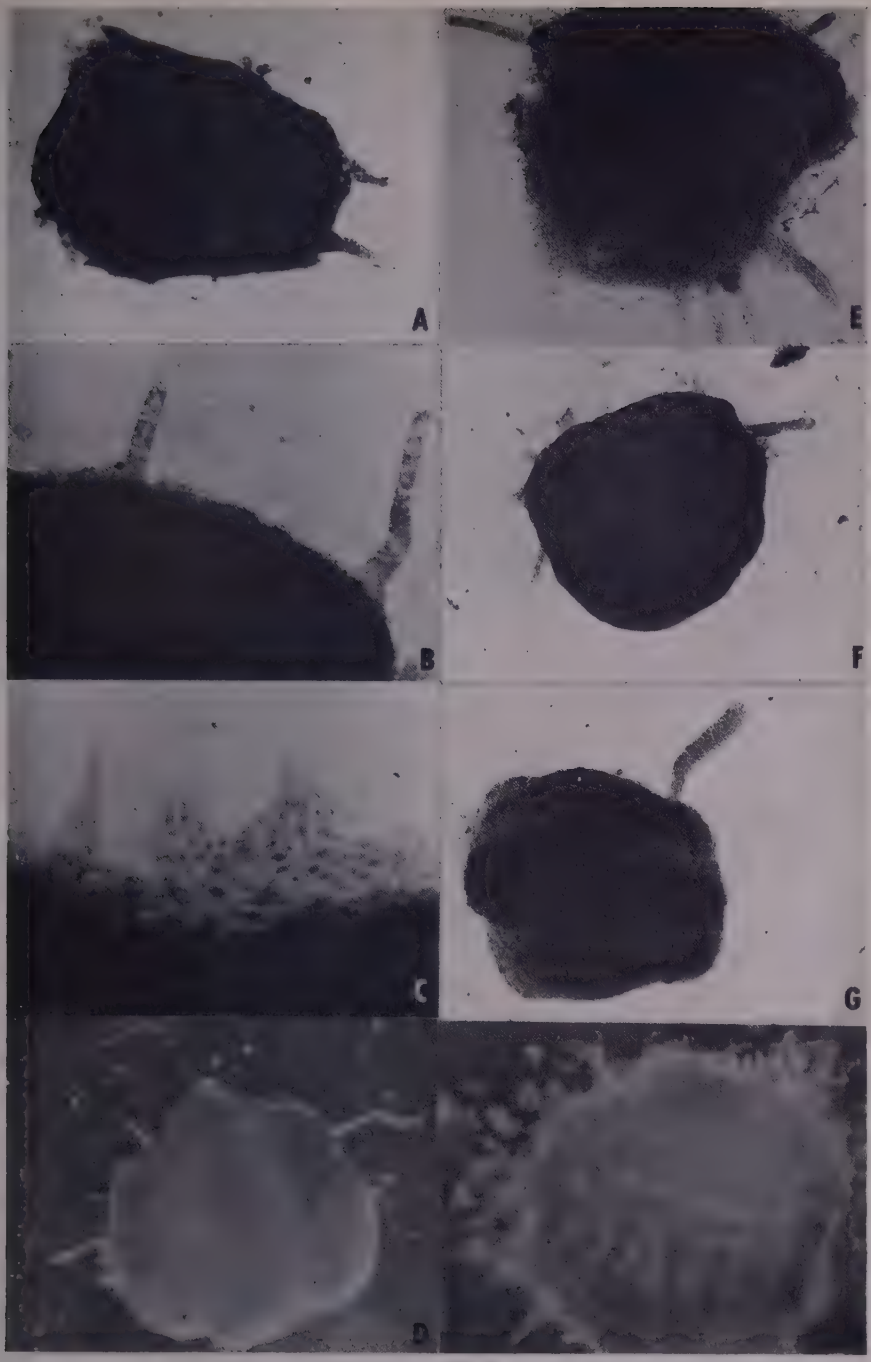
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EXPLANATION OF PLATE

FIG. A. *A. punctatum* explant in plasma clot at 2 days. Two chords of cells extend into the clot from the posterior edge of the explant on the right. Fixed preparation ×29.

FIG. B. *A.* species explant in plasma clot at 5 days. Outgrowth is limited to pronephric extension. Living preparation ×100.

FIG. C. *A. punctatum* explant at 4 days. Outgrowth on glass surface beneath clot. Fixed preparation ×100.

FIG. D. *A.* species explant at 5 days, one posterior and two lateral extensions. Posterior edge of explant is up. Living preparation ×27.

FIG. E. *A.* species explant at 7 days with six extensions. Posterior edge to the right. Fixed preparation ×22.

FIG. F. *R. pipiens* explant at day 5. A single posterior extension occurs to the right. Fixed preparation ×29.

FIG. G. *R. pipiens* explant at day 5. A single posterior extension has become flattened distally where it has grown against the glass surface. Fixed preparation ×29.

FIG. H. *A. punctatum* explant in plasma clot. By day 7, extensive migration of chromatophores and other cell types has occurred. Living preparation ×27.

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The Development of the Nervous System in Chick Embryos, studied by Electron Microscopy

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WITH SEVEN PLATES

AN electron microscope study of developing nervous tissue has been made in the chick embryo, correlating the results with those obtained by other techniques. I have been especially concerned with (1) the relationship between ectoderm and mesoderm cells at the time of neural induction, and (2) the formation of the Nissl substance.

MATERIAL AND METHODS

Tissue from 73 chick embryos incubated for periods between 1 and 13 days has been studied. Before fixation each specimen was staged according to the normal table of Hamburger & Hamilton (1951). The tissue was fixed either in osmium tetroxide, or in potassium permanganate (Luft, 1956). In either case the pH was buffered to 7·4 with sodium veronal (Palade, 1952) and fixation was at 0° C. In the early stages the whole embryo was fixed. In the later stages the nerve-cord was dissected out partially before fixation, and completely afterwards. Dehydration was with alcohols, and the specimens were then embedded either in the epoxide resin 'Araldite' (Glauert, Rogers, & Glauert, 1956) or in methacrylate. Sections were examined in a Siemens Elmiskop 1B electron microscope.

Twenty-four specimens have also been studied by light microscopy. Twelve of these were stained with silver (Bielschowsky's method) and twelve were selectively stained for Nissl substance with dahlia, cresyl violet, or neutral red.

Quantitative analysis of the cellular constituents

Twenty-five specimens at seven different stages were analysed by an adaptation of Chalkley's method (1943) as used in light microscopy. Montages were taken, each consisting of about twelve electron micrographs with magnifications

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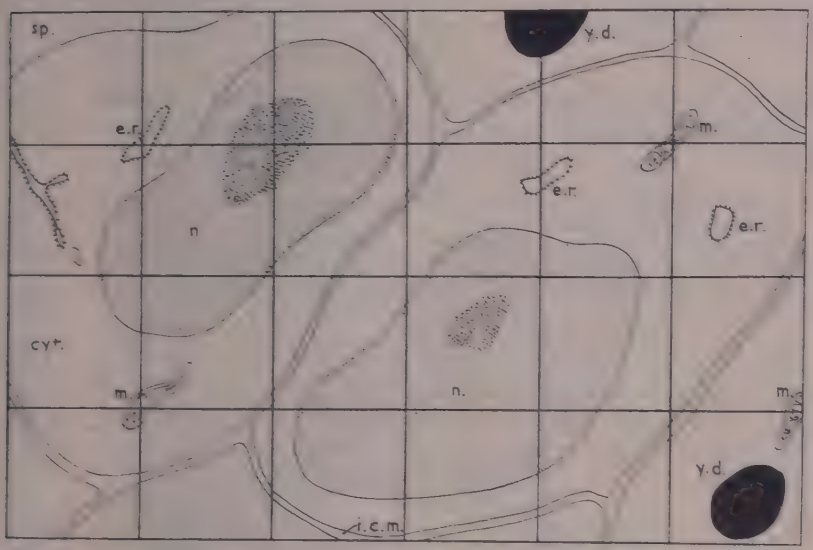
of either 35,000 or 70,000 after printing. A piece of transparent paper the same size as the photographs was marked into a grid by two sets of parallel lines at right angles to one another (see Text-fig. 1). The grid was placed over each



TEXT-FIG. 1. Diagram illustrating a method for quantitative analysis of cellular constituents in electron micrographs (adapted from Chalkley, 1943). A piece of transparent paper is marked into a grid and laid over each micrograph. The points where the grid lines intersect are used for sampling. Thirty-five such points are shown in the diagram and they coincide with the following structures: nucleus (*n.*), 5 points; mitochondria (*m.*), 3 points; membranes of endoplasmic reticulum (*e.r.*), 2 points; yolk drops (*y.d.*), 1 point; cytoplasm other than the constituents already mentioned, 21 points; extra-cellular space and intercellular matrix (*sp.* and *i.c.m.*), 3 points.

photograph and the points where the grid lines intersected were used for sampling. There were 180 such points on the grid. Counts were made of the number of sampling points which fell on the following structures in each photograph: mitochondria, endoplasmic reticulum, cytoplasm (other than the constituents already mentioned), nucleus. The process was repeated for each photograph in the montage, care being taken where two photographs overlapped not to count the same area twice. The number of points falling on the mitochondria, and of those falling on the endoplasmic reticulum (see Table 1) were then expressed for each embryo as a percentage of those falling on the 'total cytoplasm' ('total cytoplasm' has been considered as the sum of the following structures: endoplasmic reticulum, mitochondria, intra-cellular yolk drops, granules of all kinds, and the spaces between these structures. Nuclei, nuclear and cell membranes and intercellular material have not been included). The individual percentages for several embryos were then combined to give the mean percentage for each stage. Each

point shown on the graphs (Text-fig. 2) is thus derived from between about 3,000 and 6,000 readings of the sample points on the grid. The percentages of sampling points may be taken to represent the percentage amounts of mitochondrial or endoplasmic reticular substance present in the cytoplasm.

TABLE 1

Numbers of points falling on different components of the cytoplasm, according to an adaptation of the method of Chalkley for estimating areas. See Text-figs. 1 and 2

Stage	*Estimated mean age in hours*	*Specimen*	*Endoplasmic reticulum*	*Mitochondria*	*Total cytoplasm*
3¾	16	1	153	199	1,015
		2	29	44	682
		3	37	42	635
4–5	20	4	118	89	995
		5	29	67	689
		6	39	47	564
		7	78	135	1,121
6	24	8	75	161	1,106
		9	113	75	971
		10	56	27	504
10	35½	11	255	148	1,220
		12	102	130	746
		13	21	22	257
12	47	14	113	57	944
		15	154	120	1,124
		16	33	40	560
		17	89	56	520
		18	62	41	469
16–21	67	19	445	150	1,467
		20	170	104	894
		21	120	59	903
		22	164	65	1,011
31–36	190	23	371	44	1,204
		24	133	19	530
		25	417	68	1,244

Stages examined by electron microscopy

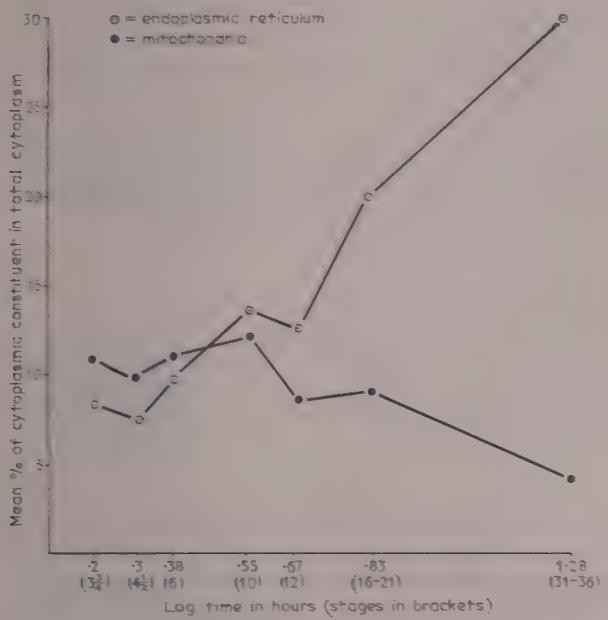
1. *The long primitive-streak stage.* This stage, as defined by Waddington (1932) and Abercrombie (1950), is later than stage 3½ of Hamburger & Hamilton (1951) and earlier than their stage 4 (Text-fig. 3 A, D).

2. *The head-process stages.* Stages 4 and 5 of Hamburger & Hamilton (1951). Stage 4 is often called the definitive primitive-streak stage and many authors have considered it to be equivalent to the long primitive-streak stage. Fig. 4 of Hamburger & Hamilton (1951) shows, however, that some of the head-process mesoderm already lies anterior to the primitive streak in stage 4 (see also Text-fig. 3 B, C, E).

3. *Stages 10–14* of Hamburger & Hamilton (1951). These stages were reached after about 40 to 48 hours' incubation (Text-fig. 3F).

4. *Stages 16–22* of Hamburger & Hamilton (1951). These stages were reached after $2\frac{1}{2}$–4 days' incubation (Text-fig. 3G).

5. *Stages 31–39* of Hamburger & Hamilton (1951). These stages were reached after 7–13 days' incubation (Text-fig. 3H).



TEXT-FIG. 2. Graph to show how the percentages of endoplasmic reticulum and of mitochondria in the cytoplasm vary during development. The way in which these percentages have been determined is described under Material and Methods (see also Text-fig. 1). Data are given in Table 1. The scatter of the points has been tested for each curve and has been found significantly non-random ($\chi_{[6]}^2 = 687$ for endoplasmic reticulum and 90 for mitochondria). The rise in the proportion of endoplasmic reticulum between stages 12 and 31–36 has been found significant ($\chi_{[1]}^2 = 220$). Similarly, the fall in the proportion of mitochondria during the same period has been found significant ($\chi_{[1]}^2 = 41$).

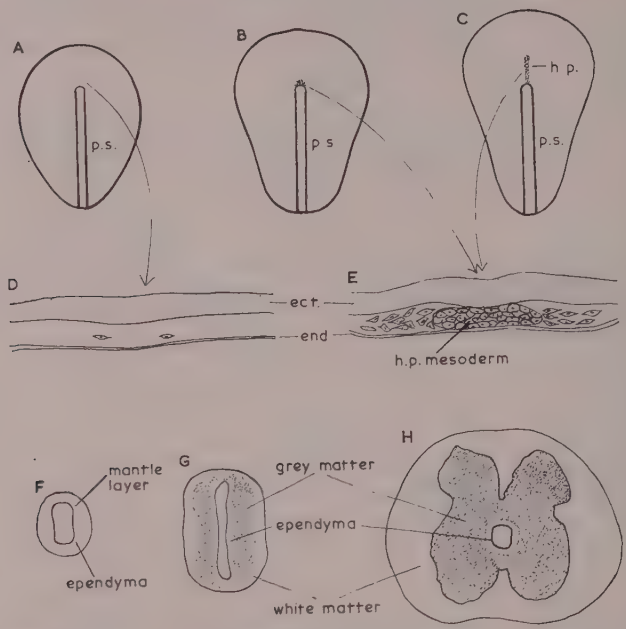
RESULTS

Early stages with special reference to neural induction

The long primitive-streak stage

The ectoderm lying anterior to the primitive streak is a pseudostratified epithelium about 30 to 40 μ in depth and may have as many as 3 or 4 nuclei between dorsal and ventral sides. It is part of the presumptive neural plate (Pasteels,

1937), but apart from a few scattered cells it is not yet underlain by mesoderm (Text-fig. 3D). It contains intra-cellular yolk droplets (Bellairs, 1958).



TEXT-FIG. 3. A, B, C, diagrams of the area pellucida. A at stage $3\frac{3}{4}$ (long primitive streak); B at stage 4 (definitive primitive streak); C at stage 5 (head-process stage). *p.s.*, primitive streak; *h.p.*, head process. D, section from A anterior to the primitive streak. E, section from B or C across the developing head process. F, G, H, sections across the nerve-cord (magnified 10 times) at stages 11 (44 hours), 17 (3 days), and 36 (10 days).

Plate 1, fig. 1 is a low-power electron micrograph of a section through the presumptive neural plate ectoderm just anterior to the primitive streak. Two endodermal cells are at the bottom of the picture; they are not in contact with the ectoderm. The section passes through the whole depth of the ectoderm and one cell can be seen to stretch from the top to the bottom of the epithelium. The ectodermal cells are separated by an intercellular matrix about 100–150 Å wide in most parts, although there are often small triangular or diamond-shaped spaces where the borders of three or more cells meet. At the dorsal surface small filamentous cytoplasmic strands covered by cell membrane often project from the free edge of the cells.

At low electron magnifications a dense line about 250 Å wide lies parallel to the cell membrane at the ventral surface of the ectoderm. It is separated from the cell membrane by a lighter zone which is about 250 Å wide. This dense line

is also found along the ventral border of the neural plate, or neural tube, in all the following stages (Plate 1, fig. 5, *d.l.*). At low electron magnifications it sometimes gives the impression of a membrane lying parallel with the cell membrane (Plate 1, fig. 5, *d.l.*). At higher magnifications, however, both the dense line and the light zone can be seen to consist of a fine granular material (Plate 1, fig. 3, *d.l.* and *l.z.*). This fine granular material differs from the cell membrane in appearance.

Between the ectoderm (or neural tube) and the mesoderm can be seen 'free material' (Plate 1, fig. 5, *f.m.*), some of which appears to be attached to the 'dense line'. At higher magnifications these can be resolved into granules, each about 100–150 Å in diameter (Plate 1, fig. 4, *gran.*). These granules, like similar ones in the cytoplasm, cannot be seen after fixation in potassium permanganate. After fixation in osmic acid they are found so intimately associated with the finer granular material of the 'dense line' that it is difficult to resolve the 'dense line' at high electron magnifications (Plate 1, fig. 4).

Most of the cells at the primitive-streak stage appear to be six- or eight-sided in section, their longest axis being usually, though not invariably, oriented dorso-ventrally (Plate 1, fig. 1). In all stages examined the nuclei are oval with two membranes which are smooth in specimens fixed in osmium tetroxide, although they are often wrinkled after fixing in potassium permanganate. Nucleoli can be seen in the large nuclei, i.e. in those which have probably been cut through the middle.

Three main types of yolk drop, including all varieties of Type 'A', are present (Plate 1, fig. 1) at the primitive-streak stage. These have been described previously (Bellairs, 1958). The sectioned mitochondria are usually oval in shape, the length seldom being greater than three times the width; their cristae are often disorganized. Both granular (Plate 1, fig. 2, *g.e.r.*) and agranular membranes of the endoplasmic reticulum (as described by Palay & Palade, 1955) are present, but usually have round or oval outlines in the sections. Occasionally the non-granular type is arranged in the way which many workers (e.g. Sjöstrand, 1956) have considered to be characteristic of the Golgi apparatus. Granules of about 100 Å or more in diameter (Plate 1, fig. 2, *gran.*) are scanty compared with the stages of 48 hours and later (see Bellairs, 1958).

Stages 4 and 5

The ectoderm is thicker at this stage, being between about 40 and 50 μ, and it seems probable that this is because more cells are present. Some cells still appear to extend through the whole thickness of the epithelium. The presumptive neural-plate region which is anterior to the primitive streak (Pasteels, 1937; Spratt, 1952) lies closely about the head-process mesoderm (Text-fig. 3E).

The principal difference between these stages and the previous one is that the cells have become more elongated, especially in stage 5. The three main types of yolk drop are still present (Plate 2, fig. 6 shows two types) although type B (fatty)

tends to be most frequent at the ependymal edge. The membranes of the endoplasmic reticulum (see Text-fig. 2) usually have circular or oval outlines (Plate 2, fig. 8 *e.r.*), which suggests that they are perhaps in the form of vesicles; but in certain especially elongated cells they appear as long pairs of membranes (Plate 2, fig. 7, *e.r.*), which indicates that they may also be in the form of tubules or sheets.

The free edge of the lowermost cells of the neural plate is covered, as in the earlier stage, with a fine granular substance, which resembles the intercellular matrix. It is in fact considered to be the basement membrane (see Discussion). Where the cells of the head-process mesoderm lie closely beneath the ectoderm this granular substance has always (10 specimens) been found to be pressed between them (Plate 2, fig. 8, *i.c.m.*). It varies in width, even in the same section, and may be as little as 200 Å or as much as 1,000 Å. Particles are present in this material (Plate 2, fig. 9, *gr.*) which resemble some of the cytoplasmic granules, as well as those of the 'free material' (see above), in the following respects: size (100–150 Å); electron density after fixation in osmium tetroxide; arrangement, which may be single or in the form of a 'rosette' (Plate 2, fig. 9, *gr.*); and in being visible after fixation in osmium tetroxide but not after fixation in potassium permanganate.

The mesodermal cells possess the same components as the ectodermal cells; they are, however, more irregular in outline, as is well known to light microscopists. Their nuclei are larger compared with the area of the cytoplasm than those of the ectoderm. It follows that the nuclei of the mesodermal cells often lie close to the cell membrane and therefore close to the ectoderm (Plate 2, figs. 6, 8, *n.m.*).

Later stages with special reference to Nissl substance and neurofibrillae

Stages 10–14

By stage 10 the neural plate has become converted into a tube along most of its length. It consists of two layers, the ependyma, which lines the neural canal and contains a large number of mitotic cells, and the mantle layer (Text-fig. 3F). At about 48 hours a few neuroblasts can be distinguished in the ventro-lateral part of the tube in silver-stained specimens; this region of the tube is about six cells deep. In the floor of the tube there is only a single layer of cells. Detailed descriptions of the developing neuroblasts at these stages are given by Cajal (1890), Tello (1923), and Hughes (1955).

All the cells are elongated with their long axes radiating out from the ependyma to the peripheral edge of the tube. Occasionally they extend as much as half-way across the wall of the tube. Some cells appear to have a short process extending out from them (Plate 4, fig. 14, *c.p.*). In some cases this may be a portion of the cell which has been sectioned obliquely. In others, however, the 'process' is characterized by possessing several paired membranes of the endoplasmic reticulum running along it (Plate 4, fig. 14, *m.e.r.*). These membranes of

the endoplasmic reticulum are packed together only in the 'processes' of these cells and seem to be more spread out in the rest of the cytoplasm. It is suggested, therefore, that the 'processes' are true ones and that the cells concerned are probably developing neuroblasts.

The lumen of the tube contains a granular substance which has probably been precipitated by fixation (Plate 3, fig. 10, *l.*). The cells are separated from one another only by an intercellular matrix about 150 Å thick.

The types of intra-cellular yolk drops have been described previously and it has been shown that their structure changes as development of the embryo proceeds (Bellairs, 1958). Those which have been classified as 'type A' drops are seldom present at this stage and have only been seen in their final form, that is when they consist entirely of 'circular bodies'. 'Complex type' yolk drops are even rarer. The 'type B' yolk drops, which are highly electron-opaque after fixation in osmic acid, are plentiful. Although they are found in all parts of the neural tube they tend to be commonest in the ependymal region (Plate 3, fig. 10, *B*).

The mitochondria are usually seen in section as round or oval bodies seldom longer than three times their width (Plate 1, fig. 5, *m.*). The small cytoplasmic particles are more numerous at this stage than in the preceding ones (see Bellairs, 1958). Neurofilaments (see below) have not been seen at this stage. The proportion of both endoplasmic reticulum and mitochondria present in the cytoplasm is shown in Text-fig. 2 and Table 1.

Stages 16–22

By stage 16 the neural tube has become closed dorsally along its whole length. In silver preparations neuroblasts can be seen in the latero-ventral parts of the tube and spinal nerves have developed. By stage 22, when more spinal nerves are visible, regions of 'grey' and 'white' matter can be recognized (Text-fig. 3G). Nissl substance cannot be seen in embryos of this stage stained with basic dyes. During this period (stages 16–22) some of the ependymal cells at the two sides of the neural tube begin to come into contact with one another in the midline, thus reducing the size of the lumen. For full descriptions of these stages see Cajal (1890) and Tello (1923).

In these and the following stages I have been concerned mainly with the ventral part of the neural tube in the anterior trunk and neck. The cells are elongated in a medio-lateral direction. They are broadest in the region of the nucleus, and in most of the sections examined their is little space between the nuclear and cellular membranes at the sides of the cell (Plate 5, figs. 19, 20, *n.m.* and *c.m.*). In some cells the distal end can be seen as an elongated process extending out towards the edge of the neural tube (Plate 5, fig. 19). Typically, where a process passes between the wide nucleated parts of two other cells it is narrower from side to side, as if it has become constricted, whereas farther laterally it is often wider as if it has been able to swell out again. Similar, though shorter, processes

have also been seen on the proximal sides of these cells. It is thought that these are genuine processes rather than parts of the cell which have been sectioned in an unusual manner, because they are not only rich in paired membranes of endoplasmic reticulum (see previous stage) but elongated sections of cells are more common in this stage than in earlier ones.

Gaps at the place where several cells meet are rare, although they are present in the 'white matter' of stage 22. In the earliest of these stages (stage 16) cells are usually separated by an intercellular matrix between 100 and 150 Å wide. By stage 21, however, the intercellular matrix has been reduced in small patches between adjacent cells to less than 50 Å wide (Plate 3, figs. 12, 13). Small villous-like processes are often packed tightly between adjacent cells (Plate 5, fig. 20). A similar condition occurs in places where the two sides of the ependyma have come together in the midline (Plate 3, fig. 11).

'Type B' yolk drops are scarce and small compared with the previous stages and with the adjacent mesoderm. No 'type A' yolk drops or 'complex' yolk drops have ever been seen in the neural tube at this stage.

The shape and appearance of the mitochondria seem to vary with the specimen, and possibly also with the fixative as noted by de Lorenzo (1957). In some embryos, especially those fixed in potassium permanganate, the mitochondria are circular or oval in section, and their length is seldom more than twice or three times their width. They have a vacuolated appearance which suggests that their cristae have been damaged. In other specimens, especially those fixed in osmic acid, longer sections of mitochondria are also seen, and in these the cristae are usually intact.

By this stage of development the proportion of endoplasmic reticulum to total cytoplasm has increased (see Text-fig. 2). The endoplasmic reticulum is most dense in the processes of the cells (Plate 5, fig. 18) where it is in two forms: (1) pairs of membranes about 150 to 200 Å apart (Plate 5, fig. 18, *m.e.r.*), and (2) circles or ovals with an internal diameter of about 250 Å (Plate 5, fig. 18, *c.e.r.*). It is suggested that these are longitudinal and transverse sections of tubules running along the cell processes. Mitochondria (Plate 5, fig. 18, *m.*) are also present in the cell processes. Neurofilaments, each about 100 Å in width, are present at this stage (Plate 5, fig. 21, *n.f.*).

In many cells the membranes of the endoplasmic reticulum are often circular or oval in outline (Plate 3, fig. 11, *e.r.*). In specimens fixed in osmic acid they are mainly of the granular type (Plate 4, fig. 15, *e.r.*). Some cells near the ependyma possess considerable amounts of endoplasmic reticulum in the form of paired membranes (Plate 3, fig. 11, *m.e.r.*); these may be cells which are developing into neuroblasts. In many of the sections of ependymal cells, however, both endoplasmic reticulum and mitochondria are scanty (Plate 3, fig. 11, *ep.*).

Stages 31–39

Dorsal and ventral horns are recognizable in the 'grey matter' from the 7th

day, and glial cells can be seen in the 'white matter' (Hughes, 1955). During the following days the nerve-cord becomes larger in transverse section; there appear to be more cells present but individual neuroblasts are also bigger. In specimens stained with basic dyes, Nissl substance can be recognized in a few cells on the 7th day, but is more conspicuous by the 10th day. During this period there is a change in shape of the lumen from a longitudinal slit to an almost square or round shape (Text-fig. 3H). This is accompanied by the development of the dorsal and ventral fissures. For a description of these stages, see Cajal (1890) and Hughes (1955). For an account of the development of the spongioblasts see Bensted, Dobbing, Morgan, Reid, & Payling Wright (1957).

Spongioblasts and glial cells. Although the present paper is not directly concerned with the development of the glial cells an attempt has been made to identify them and to distinguish them from neuroblasts. In the 'white matter' the glial cells can be identified with confidence because no neuroblast cell-bodies are present in this region (Plate 7, fig. 28, *n.g.*). These glial cells are characterized by the fact that they contain very little endoplasmic reticulum compared with the neuroblasts (see below). It is not possible, however, to use this fact for distinguishing spongioblasts and glial cells in the 'grey matter', for there are at least three types of cells in that region which have a similar appearance. They are spongioblasts, undifferentiated cells, and partially differentiated neuroblasts.

Neuroblasts. The neuroblasts are characterized by their long axons, which in favourable sections can be seen extending out from the cell-body, and by the presence of large amounts of endoplasmic reticulum in the cell-body (see below).

Axons. At least two kinds of filamentous structure appear to run along the axons.

1. *Neurofilaments (axon filaments).* These structures are about 100 Å in diameter. They have been followed for lengths of up to 2 μ and it seems possible that they may extend the whole length of the axon. Although they are mostly oriented along the axon they frequently cross one another (Plate 4, fig. 16, *n.f.*).

2. *Tubes of endoplasmic reticulum.* In longitudinal sections of axons, paired membranes can be seen running lengthwise as if tubes have been cut longitudinally (Plate 6, fig. 26). In every transverse section of an axon examined, membranes of endoplasmic reticulum can be seen in the form of circles or ovals as if tubes of the endoplasmic reticulum have been cut across (Plate 6, fig. 25). In both the longitudinal and transverse sections apparently homogeneous material is enclosed in the tubes. In some regions, however, the tubes appear to be swollen in places (Plate 6, fig. 26, *sw.*). The swollen regions are usually circular or oval. The walls of the endoplasmic reticulum tubes are about 75 Å wide. In the non-swollen regions the total width of the tubes is usually about 300 Å, but in the swollen region it may be as much as $\frac{1}{2}$ μ.

In addition to the neurofilaments and the tubes of endoplasmic reticulum, dark wavy bands, each about 150–300 Å in diameter, can often be seen running along the axon after fixation in osmium tetroxide. The number of these bands is

variable, but about five can be seen in Plate 6, fig. 23, *d.b.* It will be suggested that they are formed by the clumping together of neurofilaments, perhaps as a result of over-fixation. In the same axon, circular or oval sections of endoplasmic reticulum can be seen (Plate 6, figs. 22, 23, 24). They are between $\frac{1}{4}$ and $\frac{1}{2}\,\mu$ in diameter and are strung out in rows along the axons. Sometimes they appear to be connected by a narrow stalk (arrows, Plate 6, fig. 24). It is suggested that these may be the swollen regions of the tubes of endoplasmic reticulum and that they appear in these sections as discrete vesicles because the plane of section has not passed sagittally through the tube.

The cristae of the mitochondria found in the axons are often oriented longitudinally in the way described by Palay (1956) for adult axons (Plate 6, fig. 22, *m.*). Furthermore, the cristae seldom appear to be disrupted at this stage and the mitochondria as a whole are more electron-opaque than those in the younger specimens.

The cell-body. In the 'grey matter' the cells lie close to one another, although large gaps have occasionally been seen (Plate 7, fig. 27, *gp.*). The intercellular matrix is usually about 150 Å wide, but in patches may be as little as 50 Å. The distance between the nuclear and cytoplasmic membranes appears to have increased considerably compared with the previous stages. Much of this region of the cytoplasm is occupied by endoplasmic reticulum. This is mainly of the granular variety and is in several forms: (1) so-called cisternae, irregular in outline and as much as 4 μ in length (Plate 7, fig. 30, *e.r.* 1); (2) circular in outline, up to $\frac{1}{2}\,\mu$ in diameter (Plate 7, fig. 30, *e.r.* 2), and similar to the endoplasmic reticulum in the axons (Plate 6, figs. 22, 23, 24, *e.r.*); (3) paired membranes (Plate 7, fig. 27, *e.r.* 3, and fig. 29) which may be a stage in the formation of the Nissl substance.

Non-granular membranes of endoplasmic reticulum are also present and may be arranged in the form described as Golgi bodies by certain authors (Sjöstrand, 1956) (Plate 7, fig. 27, *e.r.* 4).

The percentage of mitochondrial substance present in the cytoplasm of the neural tube (i.e. axons, cell-bodies, and spongioblasts) was estimated (Table 1 and Text-fig. 2) and was found to be significantly less than for the preceding stages. It is possible, however, that this is due more to an increase in the amount of cytoplasm than to an actual decrease in the amount of mitochondrial substance.

DISCUSSION

Embryonic cells are constantly subjected to influences from neighbouring cells, and in some cases (e.g. during neural induction) these influences have a profound effect upon differentiation. For this reason the discussion will be divided into two main sections: (1) morphological relationships between cells, and (2) changes within the cells.

(1) *Morphological relationships between cells*

(*a*) *At the time of neural induction*

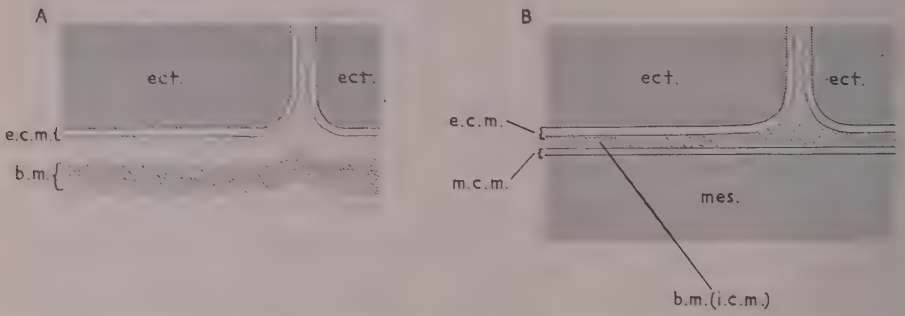
In the chick embryo the developing chorda-mesoderm is believed to be responsible for inducing neural tissue (with the possible exception of the forebrain). The evidence, which comes mainly from grafting experiments, is discussed by Waddington (1952). There are several reasons for believing that neural induction starts when the head process is beginning to develop, that is, at the definitive primitive-streak stage (stage 4 of Hamburger & Hamilton, 1951). First, it is unlikely to have taken place at the previous stage (long primitive-streak stage of Waddington, 1932), for at that time the presumptive neural plate is not yet underlain by head-process mesoderm (Pasteels, 1937), and, furthermore, the whole region is capable of extensive regulation (Abercrombie, 1950; Abercrombie & Bellairs, 1954). Secondly, neural induction has probably taken place by the time the head process is well formed, for by then the regulatory powers of the presumptive neural plate have almost entirely disappeared (Abercrombie, 1950; Abercrombie & Bellairs, 1959). It seems probable, therefore, that neural induction occurs between the two stages, that is, at the definitive primitive-streak stage when the head-process mesoderm has migrated forward beneath the presumptive neural plate (Spratt, 1952).

The present results show that at the time when neural induction is believed to be taking place the lowest cells of the ectoderm and the uppermost cells of the mesoderm are separated from one another by an intercellular matrix. In no section were the cell membranes ever found in complete contact. It must be admitted, however, that closer contact might occur for brief periods only and so be overlooked. There is, indeed, some experimental evidence that in amphibians the time needed for an induction to occur is as little as 5 minutes (Nieuwkoop, 1955).

Eakin & Lehmann (1957) reported having seen in electron micrographs of amphibian gastrulae, at the time of neural induction, regions between the ectoderm and mesoderm where the cell membranes appeared to have broken down. They suggested that these might be regions of anastomosis, but, as they themselves pointed out, higher magnifications and different fixatives will be necessary before this interpretation can be accepted.

The intercellular matrix found between the ectoderm and mesoderm in the present investigation appears to be derived mainly from the basement membrane of the ectoderm. The evidence is as follows. At low electron magnifications a dense line can be seen lying parallel with the free edge of the cells at the base of the ectoderm or neural tube (Plate 1, fig. 5). This was found at all stages examined except at the time when induction was believed to be in progress (Plate 2, fig. 8). This dense line has also been reported by Duncan (1957) in this region. At higher electron magnifications, however, it can be seen that the dense line is not in fact part of the cell membrane, but that it consists of a

fine granular material (Plate 1, fig. 3). Furthermore, it is separated from the cell membrane by a similar, though less dense, region of granulation. These findings are in agreement with those of Robertson (1957) on the structure of the free surfaces of nerve- and muscle-fibres. He has also shown that this granular material is present along the base of a large number of different cell types which are known from light microscopy to possess a basement membrane. He has concluded, therefore, that the granular material is in fact the basement membrane.



Text-fig. 4. Diagram to illustrate the relationship between ectoderm and mesoderm during neural induction. A, basal edge of two ectodermal cells of the presumptive neural plate prior to neural induction (see Text-fig. 1 A, D). *ect.*, ectodermal cell; *e.c.m.*, cell membrane of ectodermal cell; *b.m.*, 'basement membrane', composed largely of granular material. B, basal edge of the same cells when they have become underlain by mesoderm, that is during neural induction (see Text-fig. 1 B, C, F). *mes.*, mesodermal cell; *m.c.m.*, cell membrane of mesodermal cell; *b.m.* (*i.c.m.*), the former basement membrane of the ectodermal cells which has now become pressed into an intercellular matrix between the ectoderm and mesoderm cells.

This granular material of the basement membrane is present along the base of the presumptive neural plate ectoderm before the time of induction (Plate 1, fig. 4). It is suggested, therefore, that it contributes to the intercellular matrix which lies between the ectoderm and mesoderm cells at the time of induction. It appears to be compressed during this period, for whereas the fine granular material along the free edge of the ectoderm cells was usually about 400–600 Å wide, the intercellular matrix between the ectoderm and the mesoderm cells was sometimes found to be as little as 200 Å wide (see Text-fig. 4).

(*b*) *During neuroblast formation*

When the embryo is between the stages of 16 and 21 (about 50 hours to $3\frac{1}{2}$ days) many of the neuroblasts have entered the 'grey matter' and are beginning to differentiate. It has not been possible to recognize the developing glial cells at this stage, either by light or electron microscopy.

In most regions of the neural tube the cells are separated by an intercellular matrix of 100–150 Å in width. In some regions in the chick spinal cord, however, the intercellular matrix is considerably reduced, the two adjacent membranes

appearing to be almost in contact. It is suggested that changes at the surface between adjacent cells might serve to anchor these cells, which are probably neuroblasts, and prevent them from migrating into the 'white matter'. This possibility is supported by the fact that anchoring is known to occur in amphibian neuroblasts growing in tissue culture, the posterior end becoming attached to the substrate while the free anterior end continues to move forward (Holtfreter, 1947). This possibility that membranes might become anchored to one another by a reduction of the intercellular matrix was suggested by Robertson (1958).

(2) *Changes within the cells*

(*a*) *Intra-cellular yolk drops*

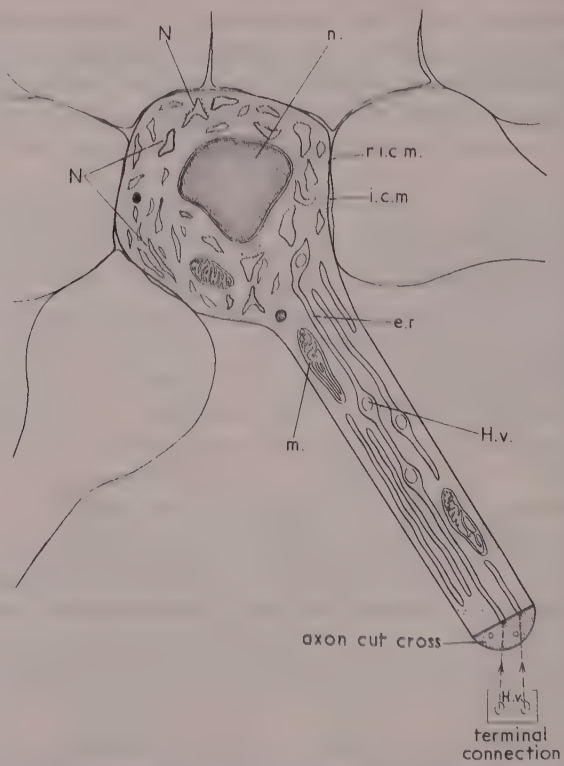
The structure and fate of the intra-cellular yolk drops has been discussed in a previous paper (Bellairs, 1958). By the stage of about 48 hours most of the yolk drops have disappeared from the neural tube; this corresponds with the time at which the blood has begun to circulate and at which partially digested yolk is being brought via the vitelline veins from the yolk sac (Schechtman, 1956). Some of the fatty type of yolk drops (type B) remain, however, and have been seen as late as stage 16 (see also fig. 6 of Duncan, 1957).

It has been shown that various structural changes occur in the mitochondria as the neuroblasts develop. These are: (1) a change from circular or oval to more elongated outlines; (2) a reduction in the disruption of the cristae; and (3) an increase in electron opacity after fixation in osmium tetroxide. It is possible that these changes may reflect alterations in the physiological activity in the nervous system. Indeed, Moog (1952) has shown that during this period there is an alteration in the relative activity of various enzymes in the embryo, some of which (e.g. cytochrome oxidase) are known to be associated with mitochondria (Gustafson, 1954).

(*b*) *Endoplasmic reticulum and Nissl substance*

The characteristic feature of all neurones is the presence in their cytoplasm of Nissl substance. Palay & Palade (1955) have equated the Nissl substance with endoplasmic reticulum of the type which possesses granules attached to its membranes. The present paper provides the following evidence in favour of Palay & Palade's interpretation. (1) There is a rise in the percentage of endoplasmic reticulum in the cytoplasm of the developing neuroblasts during the stages examined (Text-fig. 2). The greatest increase is between the 4th and 10th days (about stages 23–36) which is the time when the Nissl substance is known to be developing (Hughes, 1955). (2) It is typical of Nissl substance to be arranged around the nucleus, and it is in this region that the increase in endoplasmic reticulum chiefly occurs (cf. Plate 7, fig. 27 and Plate 5, fig. 19). (3) Although the disposition of the membranes of endoplasmic reticulum into the parallel sheets or lamellae which Palay & Palade described as typical for Nissl substance was

seldom seen in my specimens, certain configurations (Plate 7, fig. 27, *e.r.* 3, and Plate 7, fig. 29) which may be stages in the formation of Nissl substance became



TEXT-FIG. 5. Interpretative diagram of a neurone of a chick embryo at about 10 days' incubation. The whole cell is cut transversely but the axon is also cut through vertically and its terminal connexion is indicated by broken arrows. The intercellular matrix between the cell-body and the neighbouring cells is reduced in small patches; this may help to anchor the cell-body in the grey matter. Endoplasmic reticulum covered with osmiophilic granules forms the Nissl substance in the cell-body. Tubes of endoplasmic reticulum run along the axon. (In any section of this sort only a few of the tubes will be exposed along their whole length.) Water droplets (Hughes's 'vacuoles') are taken in at the tip of the axon and may pass up the tubes of endoplasmic reticulum to the cell-body (see Discussion). *H.v.*, Hughes's 'vacuoles'; *i.c.m.*, intercellular matrix; *r.i.c.m.*, reduced intercellular matrix; *m.*, mitochondrion; *N.*, developing Nissl substance; *n.*, nucleus. The neurofilaments are not shown.

prominent about the 10th day. Similarly, the basophilia of these cells as seen by light microscopy was not conspicuous until about the 9th to 10th days.

As the Nissl substance forms there is a great increase in cell volume (Hydén, 1943). There is evidence (see below) that this may be brought about, at least in part, by new material entering the neurone at the tip of the axon and passing up

to the cell-body, possibly via tubes of endoplasmic reticulum (see Text-fig. 5). The nucleus plays a large part in the formation of proteins in the nerve cell-body (Hydén, 1943). It is possible, therefore, that the Nissl substance or its precursor is formed by the action of the nucleus on this new material. The evidence that new material may enter the cell-body in this way is as follows:

(1) Droplets of culture medium are taken in at the tips of axons growing in tissue culture. This was shown in films taken by Hughes (1953), whose material was from chick spinal ganglia of the same stage of development (7–12 days). He has called these droplets 'vacuoles'. Hughes found that in some cases the 'vacuoles' passed up the axon to the cell-body.

(2) The circular structures which I have seen in the axons (Plate 6, figs. 22, 23, 24) are of comparable size (up to $\frac{1}{2}\,\mu$) to Hughes's 'vacuoles' (up to $\frac{2}{3}\,\mu$). They are usually of the same shape (round) although occasionally they are more elongated. It is concluded that they may be the same structures as Hughes's 'vacuoles'. Similar, though smaller, structures have however recently been seen in the dendrites of adult neurones (Gray, unpublished).

(3) In some favourable sections these 'vacuoles', which lie one behind the other in the axons, appear to be connected by a narrow stalk (Plate 6, fig. 24). Tubes of the endoplasmic reticulum have also been shown in the present investigation to run along the axons. In places these tubes are swollen (Plate 6, fig. 26, *sw.*) and it is suggested that these swellings may be caused by Hughes's 'vacuoles' lying in the tube. Similar tubes with smaller swellings of the same type have also been reported in adult neurones (Palay, 1956, 1957).

A little granular endoplasmic reticulum is present in the cells from at least as early as the primitive-streak stage (Plate 1, fig. 2), and it seems reasonable to suppose that this might act as a pattern for the building up of similar material. There is also the possibility tentatively suggested by Palay & Palade (1955) that the non-granular endoplasmic reticulum might give rise to the granular.

There can be little doubt that many of the axons have already made their terminal connexions by the stage of 9 days' incubation, for spontaneous activity occurs in chick embryos from the 5th day or earlier (Windle & Orr, 1934; Kuo, 1932). It is possible, therefore, that the contents of Hughes's 'vacuoles' vary according to the axon connexions, and this factor could lead to variations in the differentiation of the cell-bodies. This might account to some extent for the well-known effect that terminal connexions have on the development of the cell-body (Hamburger & Levi-Montalcini, 1950; Hughes & Tschumi, 1957, 1958).

No evidence is yet available to show whether or not the tubes of endoplasmic reticulum found in each axon are continuous with the cell membrane at the tip of the axon. This possibility has been suggested for other types of cells (Palade, 1956). A discussion of the various concepts of how pinocytosis takes place (for example, see Bennett, 1956) will be more appropriate when this information has been obtained.

The nature of the neurofibrillae

Different authors have applied the term neurofibrillae to a variety of structures (see Hughes, 1954). A suitable definition might be that they are long, apparently continuous threads visible by light microscopy after silver staining. It is generally assumed that the silver acts by becoming deposited on the surfaces of membranes or similar structures. It is suggested as a result of the present investigation that there are two sets of structures in the embryonic neurone on which silver could become deposited.

1. *The neurofilaments (axon filaments).* These structures are about 100 Å in diameter (Plate 4, fig. 16, *n.f.*). They have been seen in electron micrographs soon after the neurofibrillae first became stainable with silver (stage 16). They have not been seen before this stage, although it is possible that a sufficiently wide search has not been made. They are present in adult neurones (Fernández-Morán, 1952; Palay & Palade, 1955) and correspond in size to similar structures recorded in living material by polarized light (Bear, Schmitt, & Young, 1937). For this reason it is considered that they are not fixation artefacts. It has been suggested that although individual filaments are too small to be resolved by ordinary light microscopy, aggregates of them encrusted with silver would be visible (Fernández-Morán, 1952; Palay & Palade, 1955). This is supported by the fact that in some specimens in the present investigation, ill-defined dark bands are visible which are between 150 and 300 Å wide. That is, they are too big to be single neurofilaments (which are about 100 Å wide) and too small to be tubes of the endoplasmic reticulum (which are about 300 to 500 Å wide). It is suggested that they are neurofilaments which have become clumped together, perhaps through over-fixation. Similarly, Couteaux (1956) found that the neurofilaments in the nerves of *Hirudo medicinalis* could be made to clump together by altering the fixative.

2. *Tubes of endoplasmic reticulum.* I have shown in this investigation that tubes of endoplasmic reticulum are present in the axons. They are of comparable width (up to about $\frac{1}{2}\mu$) to the neurofibrillae in silver preparations of chick embryos of the same stage of development. Moreover, they appear to run continuously along the axons in the same way as the neurofibrillae. Endoplasmic reticulum is also plentiful in the cell-body at this stage. Its structure as seen in sections has been described above. It is possible, however, that if a reconstruction of serial sections were made, these apparently separate units of endoplasmic reticulum would be found to be continuous with one another. Thus a continuous system of paired membranes and tubes would be present. It is suggested that this would provide a second set of surfaces upon which silver might be deposited during silver staining. Basic dyes, however, would probably stain the ribonucleoprotein granules attached to these membranes (Palay & Palade, 1955). In some photographs the walls of the endoplasmic reticulum appear to be linked by a narrower region about 75 to 100 Å wide (Plate 4, fig 17, arrow). It is possible that

this narrow part is the edge of the wall of a connecting piece of endoplasmic reticulum which has been merely grazed by the sectioning. Alternatively, it is possible that the narrow regions are neurofilaments and that the endoplasmic reticulum and the neurofilaments are different phases of the same structure. In favour of the second suggestion is the fact that in *H. medicinalis* the neurofilaments, which are larger than in vertebrates (200 Å wide as opposed to about 100 Å), appear to have a tubular structure (see Couteaux, 1956, figs. 3 and 5).

SUMMARY

1. Developing neural tissue has been studied by electron microscopy in 73 chick embryos ranging from the primitive-streak stage to 13 days of incubation.

2. At the time of neural induction (definitive primitive-streak stage) an intercellular matrix between 200 Å and 1,000 Å wide was always found between the ectoderm of the presumptive neural plate and the underlying mesoderm. Small particles, each about 100 to 150 Å in diameter, could be seen in this matrix. Evidence is presented to show that this intercellular matrix is derived, at least in part, from the basement membrane which lies along the basal edge of the ectoderm in the preceding stage.

3. From about 3½ to 4 days' incubation patches have been seen around the base of developing axons in which the intercellular matrix is reduced in width. It is suggested that this serves to anchor the neuroblast cell-body in the mantle layer.

4. The developing neuroblasts possess tubes of endoplasmic reticulum running along their axons. Structures which are interpreted as swellings of these endoplasmic reticulum tubes can be seen in the axons between the 7th and the 13th days. It is suggested that the tubes may form a channel up which the 'vacuoles' taken in by pinocytosis (previously described by Hughes, 1953) pass to the cell-body.

5. An adaptation of Chalkley's method (1943) has been used for estimating the amounts of the endoplasmic reticulum, mitochondria, yolk drops, and other cytoplasmic constituents in relation to the cytoplasm. By this method it has been shown that there is an increase in the proportion of endoplasmic reticulum in the cytoplasm as the neuroblasts develop. This is attributed to the formation of tubes in the axon, and to the development of Nissl substance in the cell-body.

6. The proportion of the mitochondria in the cytoplasm becomes reduced between the 4th and the 10th days, as the proportion of endoplasmic reticulum rises.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Transverse section through presumptive neural plate ectoderm at the long primitive-streak stage (stage $3\frac{3}{4}$) (see Text-fig. 1 A, D). A cell on the left stretches from the top to the bottom of the epithelium. Two endoderm cells (*en.*) are at the bottom of the picture. *n.*, nucleus; *A*, *B*, and *C*, three types of yolk drops; *c.m.*, cell membrane. Osmic acid fixation. Magnification ×4,700.

Fig. 2. Section through an ectoderm cell in the presumptive neural plate at the long primitive-streak stage (stage $3\frac{3}{4}$). *g.e.r.*, membranes of endoplasmic reticulum with granules attached to them; *gran.*, granules about 100 Å in diameter in the cytoplasm. Osmic acid fixation. Magnification ×54,000.

Fig. 3. Section through the ventro-lateral part of the neural tube in the cervical region of an embryo at stage 21. The free basal edge only is shown. *cyt.*, cytoplasm of the cell; *c.m.*, cell membrane; *d.l.*, dense line parallel with the cell membrane; *l.z.*, light zone. Potassium permanganate fixation. Magnification ×130,000.

Fig. 4. Section through the base of the neural plate ectoderm at the long primitive-streak stage (stage $3\frac{3}{4}$, see Text-fig. 1 A, D). Granules, each about 100 to 150 Å, are attached to and perhaps intermingled with the fine granular material seen in Fig. 3. *cyt.*, cytoplasm of the cell; *c.m.*, cell membrane; *d.l.*, dense line; *gran.*, granules. Osmic acid fixation. Magnification ×111,000.

Fig. 5. Section through the ventro-lateral part of the neural tube in the anterior trunk region of an embryo at stage 12. The basal edge of the epithelium is on the right. *d.l.*, dense line parallel with the cell membrane; *c.m.*, cell membrane; *f.m.*, free material between neural plate and mesoderm; *m.*, mitochondrion. Osmic acid fixation. Magnification ×16,000.

Plate 2

Fig. 6. Transverse section through the presumptive neural plate ectoderm at the earliest head-process stage (stage 4; see Text-fig. 1 B, C, E). This electron micrograph is part of a montage. At the bottom of the picture a mesoderm cell is pressed against two ectoderm cells; the region of contact is indicated by arrows. *A*, type A yolk drop; *B*, type B yolk drop; *n.e.*, nucleus of ectoderm cell; *n.m.*, nucleus of mesoderm cell; *e.r.*, endoplasmic reticulum. Osmic acid fixation. Magnification ×4,700.

Fig. 7. Endoplasmic reticulum (*e.r.*) consisting of elongated paired membranes, present at the head-process stage (stage 5). *B*, type B yolk drop with the typical empty appearance after this fixative. Potassium permanganate fixation. Magnification ×13,800.

Fig. 8. Region of 'contact' between the ectoderm and the underlying mesoderm at the earliest head-process stage (stage 4). This region is comparable to that in Fig. 6. *e.*, ectoderm cell; *mes.*, mesoderm cell; *n.m.*, nucleus of mesoderm cell; *i.c.m.*, intercellular matrix; *e.r.*, endoplasmic reticulum; arrows indicate the cell membranes of the ectoderm and mesoderm cells respectively. Osmic acid fixation. Magnification ×30,800.

Fig. 9. Enlargement of region of 'contact' similar to the one in Fig. 8 to show granules (*gr.*) in the intercellular matrix. Arrows indicate the cell membranes of the ectoderm and mesoderm cells respectively. *e.*, ectoderm cell; *m.*, mesoderm cell. Osmic acid fixation. Magnification ×46,200.

Plate 3

Fig. 10. Ependymal edge of the hind-brain region at stage 12. *l.*, lumen of tube containing granular material; *B*, type B yolk drop. Osmic acid fixation. Magnification ×3,500.

Fig. 11. Ependymal edge of neural tube in the cervical region (stage 17). This picture is part of a montage across the whole width of the neural tube. Arrows show where the two sides of the lumen have come into contact with each other in the midline. *ep.*, ependymal cells; *m.e.r.*, paired membranes of endoplasmic reticulum; *m.*, mitochondrion; *l.*, remains of lumen. Potassium permanganate fixation. Magnification ×11,500.

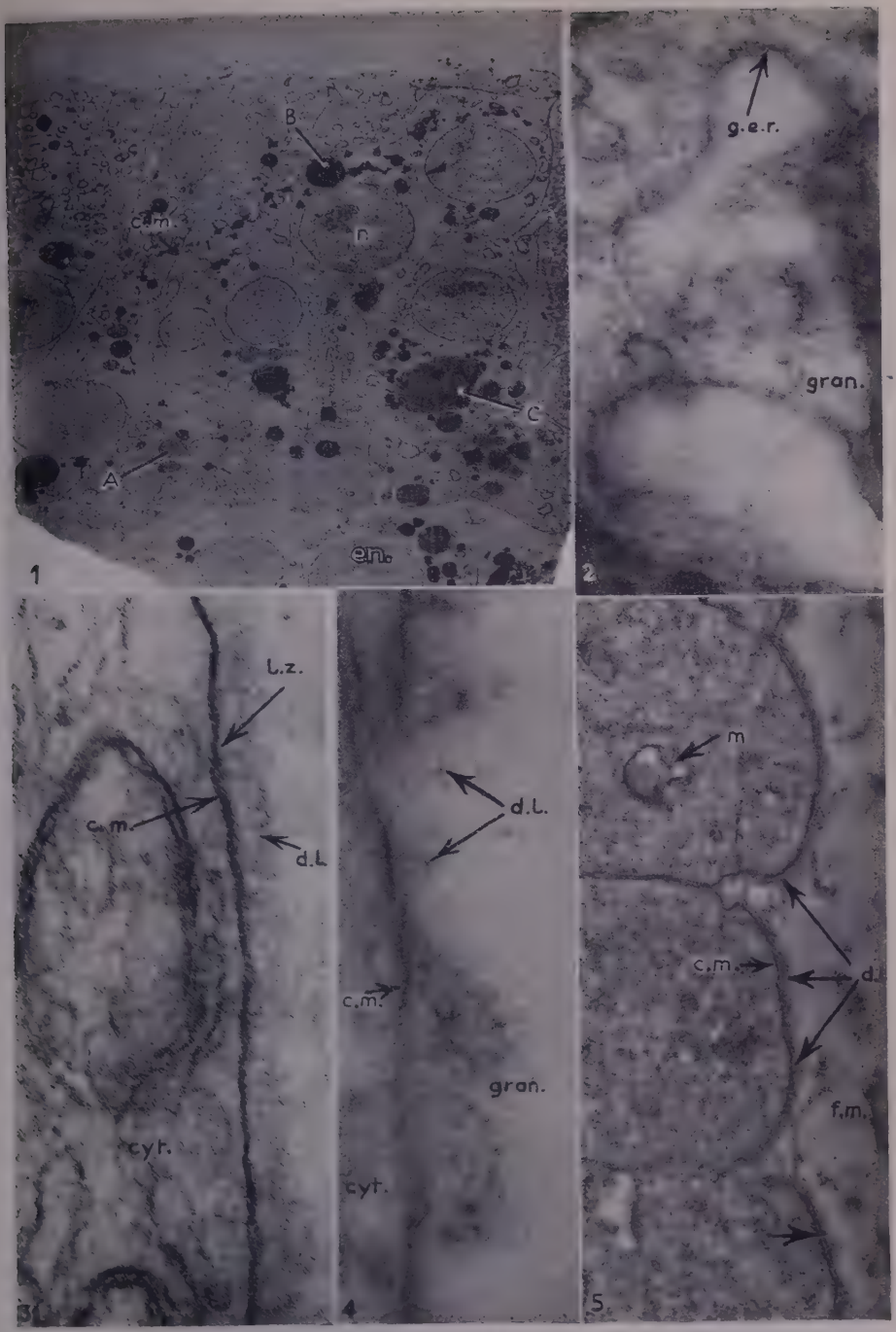
Figs. 12 and 13. Junction of the cell-bodies of two cells at stage 21. Cell membranes of adjacent cells are indicated by arrows. *i.c.m.*, intercellular matrix. In Fig. 13 the intercellular matrix is present only at the top of the picture; in the lower part of the picture it is reduced or absent, the two membranes being pressed together. Potassium permanganate fixation. Magnification ×46,200.

Plate 4

Fig. 14. Section through the ventro-lateral part of the neural tube in the anterior trunk region of an embryo at stage 11. This photograph, which is part of a montage, shows a process (*c.p.*) extending from a cell which is probably a neuroblast. The cell process possesses several paired membranes of the endoplasmic reticulum running along it (*m.e.r.*) (see text). The main part of the cell is not shown but lies beyond the top of the page. Potassium permanganate fixation. Magnification ×11,500.

Fig. 15. Membranes of endoplasmic reticulum with granules attached (*e.r.*) from a cell in the anterior trunk region of the neural tube at stage 18. Osmic acid fixation. Magnification ×30,500.

Fig. 16. Section through the ventro-lateral part of the neural tube in the cervical region of an



R. BELLAIRS

Plate 1

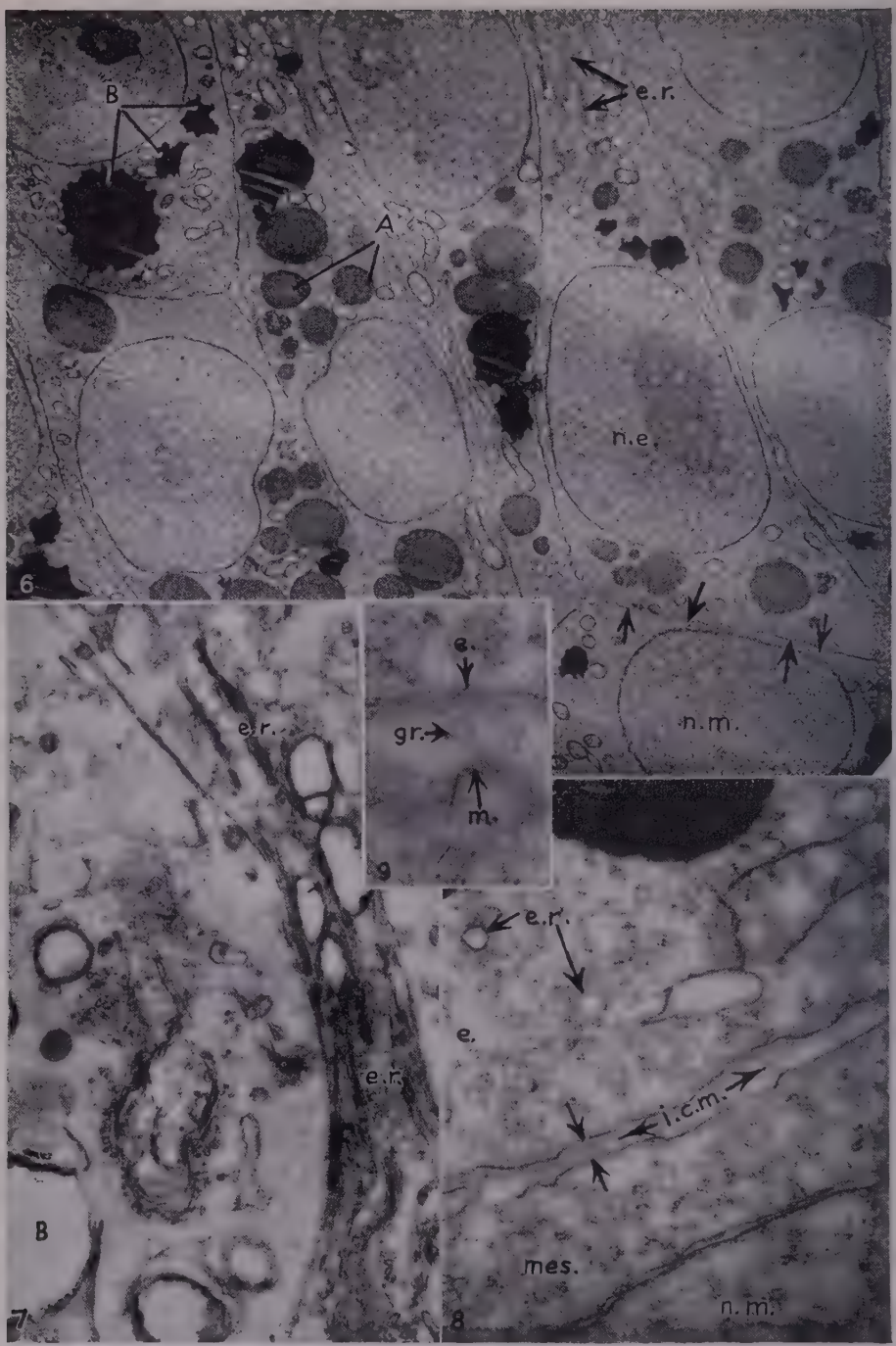


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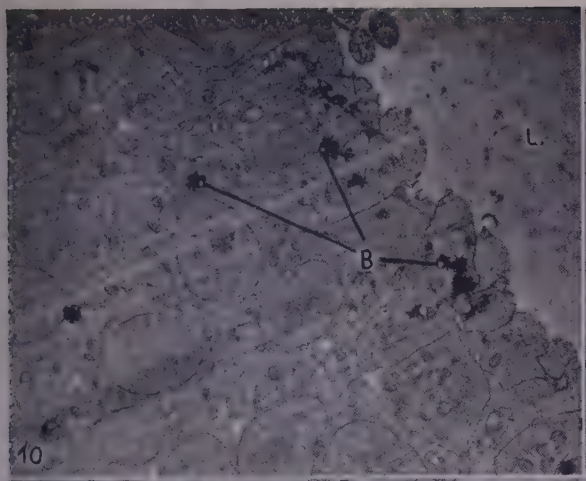
Plate 2



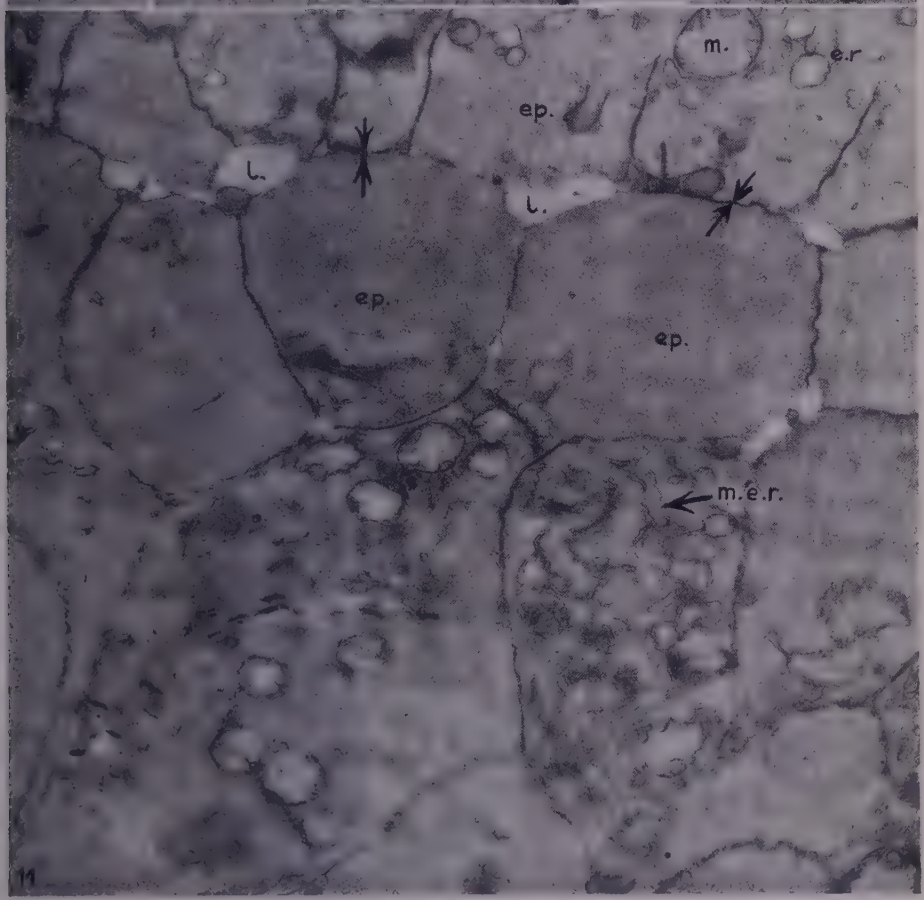


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Plate 3

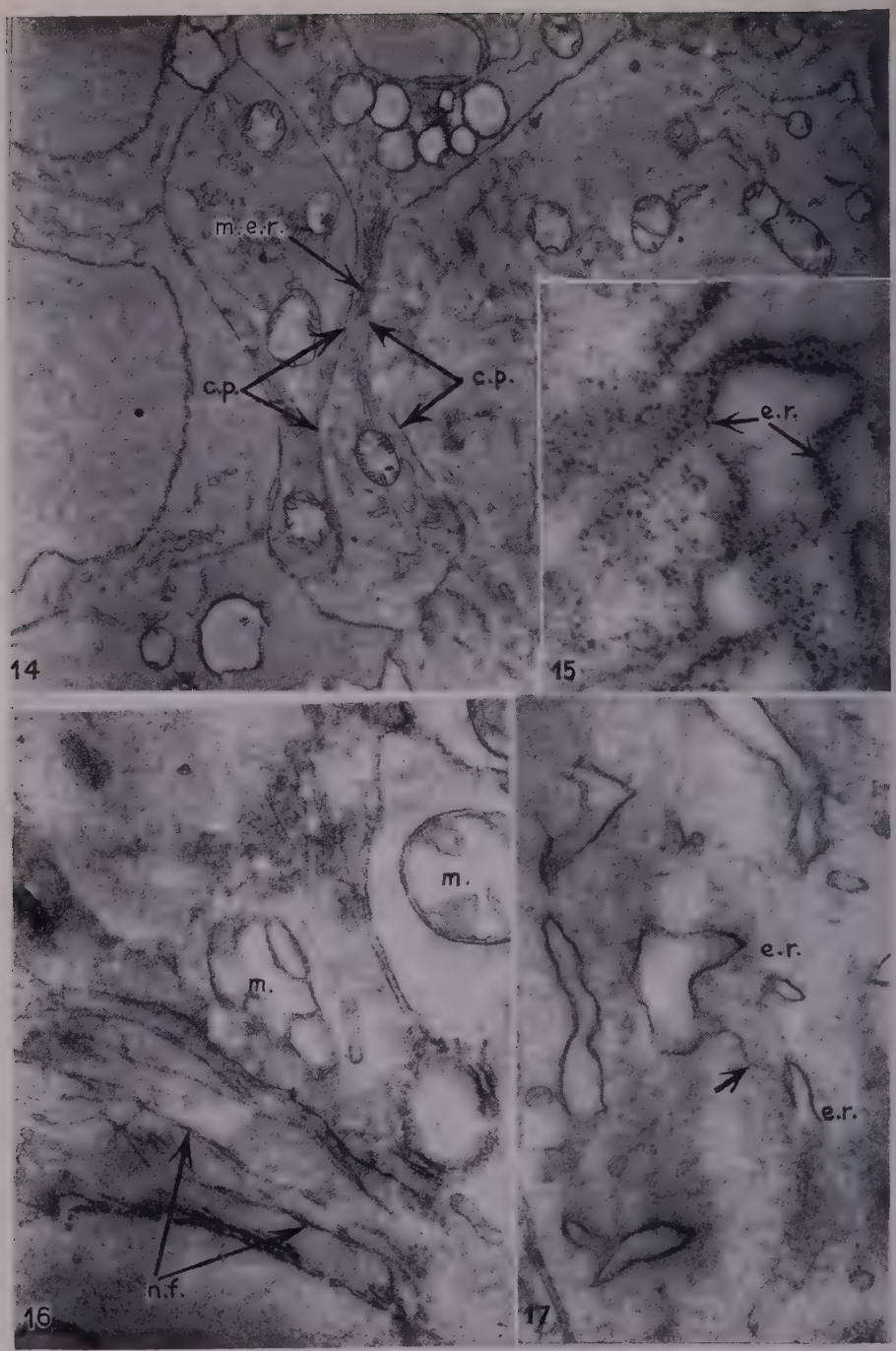

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Plate 4

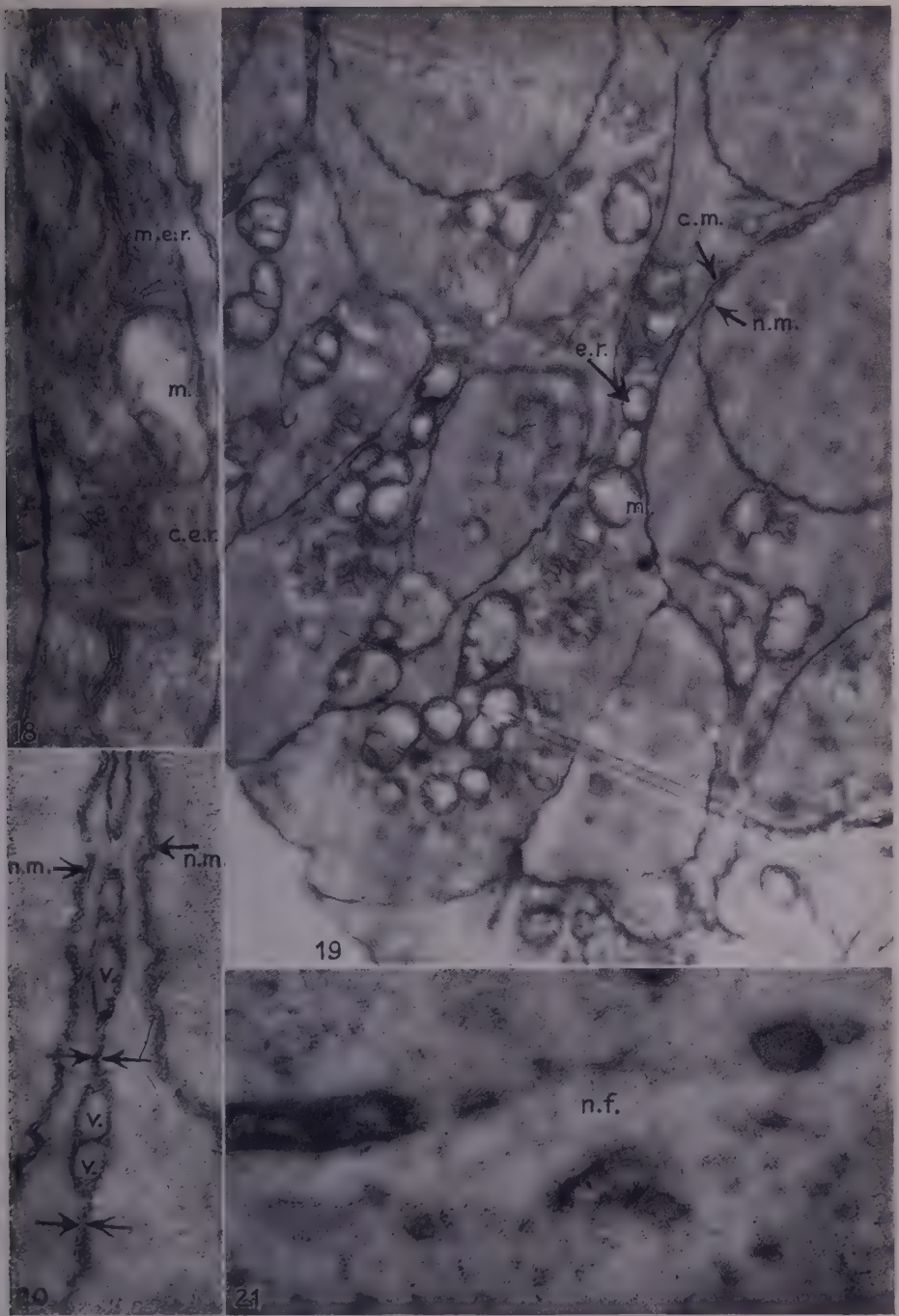

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Plate 5

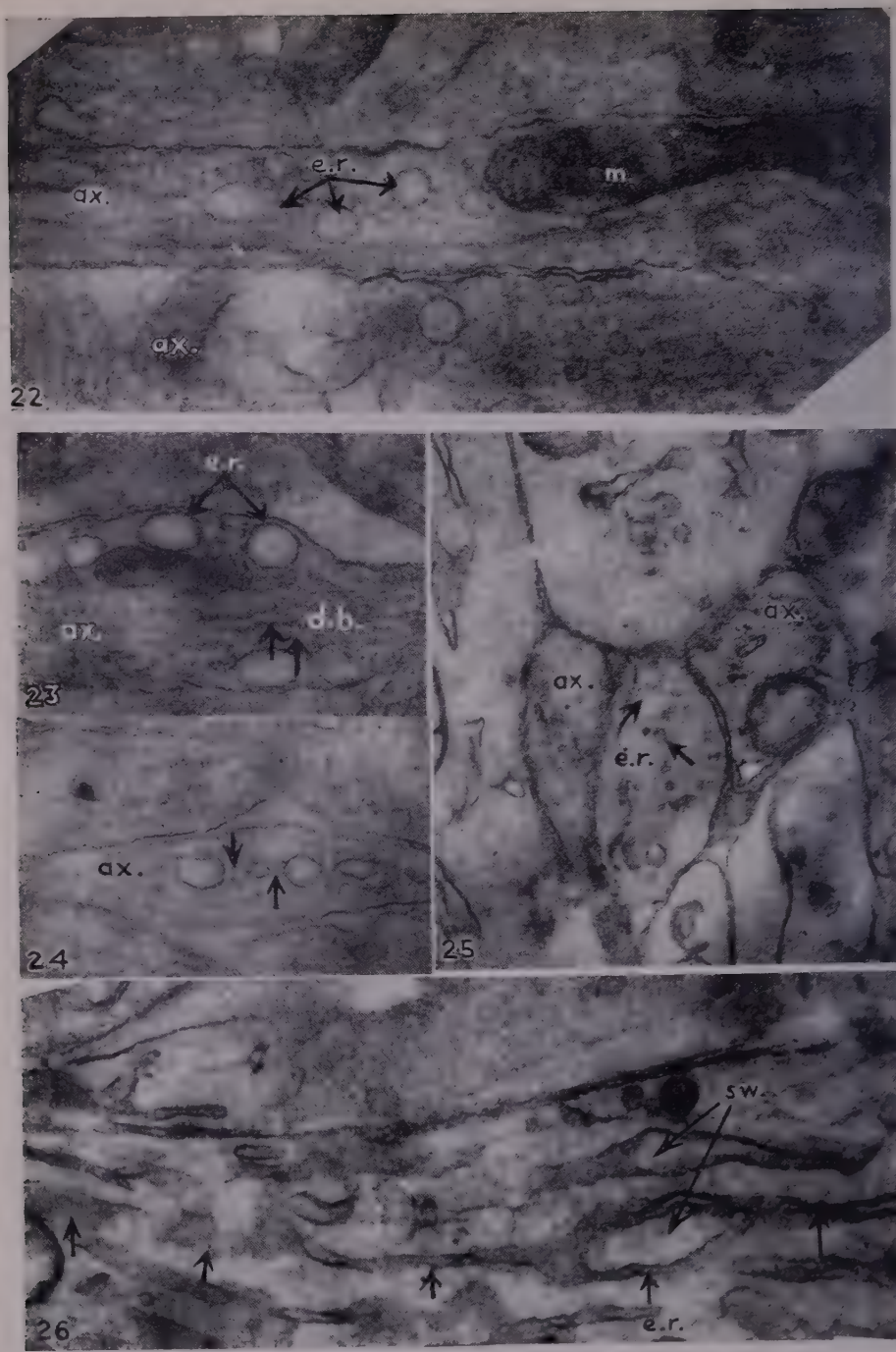

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Plate 6

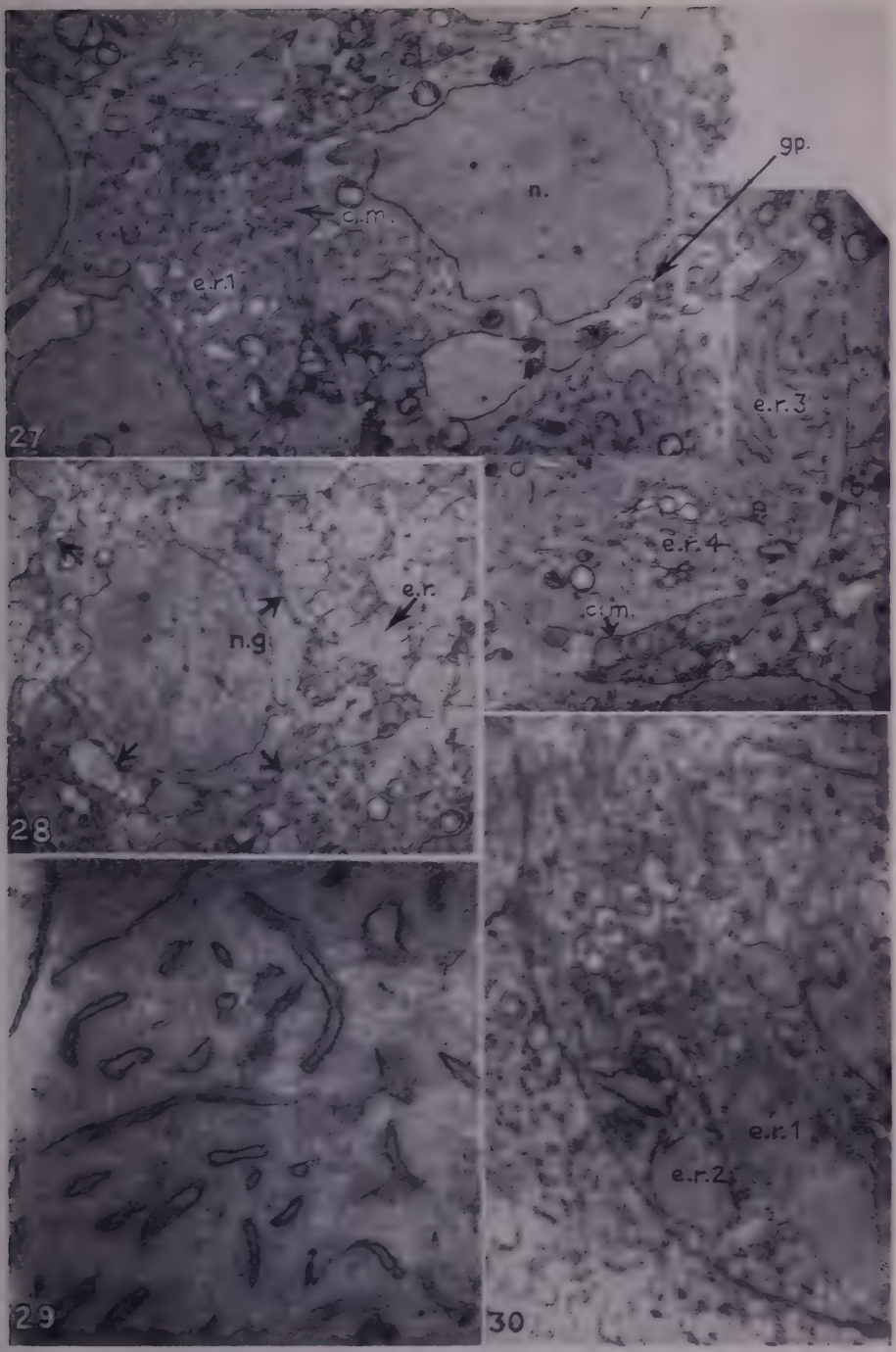

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Plate 7

embryo at stage 36. In the lower part of the picture the section passes longitudinally through an axon. *n.f.*, neurofilaments; *m.*, mitochondrion. Potassium permanganate fixation. Magnification ×38,500.

FIG. 17. Endoplasmic reticulum in the cell-body of a neuron from an embryo at stage 34. Two profiles of endoplasmic reticulum (*e.r.*) are joined by a narrower region (arrow). Potassium permanganate fixation. Magnification ×38,500.

PLATE 5

FIG. 18. Longitudinal section of an axon from a neuroblast (cervical region, stage 17). *m.e.r.*, paired membranes of endoplasmic reticulum; *c.e.r.*, circles of endoplasmic reticulum; *m.*, mitochondrion. Potassium permanganate fixation. Magnification ×23,100.

FIG. 19. Peripheral edge of neural tube in the cervical region at stage 16. An elongated process extends to the edge of the neural tube (bottom left) from a cell whose nucleus lies at the top right-hand corner of the photograph. *n.m.*, nuclear membrane; *c.m.*, cell membrane; *e.r.*, membranes of endoplasmic reticulum; *m.*, mitochondrion. Potassium permanganate fixation. Magnification ×12,650.

FIG. 20. The edges of two adjacent cells (stage 20) in the cervical region of the neural tube. They are separated in places by small villous processes of other cell(s). *v.*, villous process cut in transverse section; arrows indicate the cell membranes of the two adjacent cells; *n.m.*, nuclear membranes. Potassium permanganate fixation. Magnification ×30,300.

FIG. 21. Meshwork of neurofilaments (*n.f.*) present in the cell process of a neuroblast at stage 22. Osmic acid fixation. Magnification ×20,700.

PLATE 6

FIGS. 22, 23, and 24. Longitudinal sections of axons from the white matter of the cervical region at stage 35. *ax.*, axon; *d.b.*, dark bands; *m.*, mitochondrion; *e.r.*, endoplasmic reticulum. Osmic acid fixation. Magnification ×26,000.

FIG. 25. Transverse sections across the white matter from the cervical region at stage 36. *ax.*, axon; *e.r.*, small circles of endoplasmic reticulum in each axon; they are tubes cut across (cf. Fig. 26). Potassium permanganate fixation. Magnification ×30,800.

FIG. 26. Longitudinal section of an axon to show paired membranes of endoplasmic reticulum (indicated by arrows). They are swollen in places (*sw.*). Potassium permanganate fixation. Magnification ×38,500.

PLATE 7

FIG. 27. Transverse section across the 'grey matter' in the cervical region at stage 36. *n.*, nucleus; *c.m.*, cell membrane; *e.r.* 1, *e.r.* 3, and *e.r.* 4 are different types of endoplasmic reticulum (see text); *gp.*, gap between axons. Potassium permanganate fixation. Magnification ×6,900.

FIG. 28. Transverse section across a glial cell (*n.g.*) in the 'white matter' in the cervical region at stage 36. Note the sparseness of endoplasmic reticulum in this cell. The borders of the cell are indicated by arrows. Around the glial cell are many axons cut in transverse section, and in each of these, transverse sections of endoplasmic reticulum can be seen as small dots (*e.r.*). Potassium permanganate fixation. Magnification ×5,750.

FIG. 29. Endoplasmic reticulum in the cell-body of a neuron (stage 35) arranged as paired membranes. Potassium permanganate fixation. Magnification ×23,100.

FIG. 30. Part of the cell-body of a neuron (cervical region, stage 27), *e.r.* 1 and *e.r.* 2, endoplasmic reticulum (see text). Osmic acid fixation. Magnification ×19,250.

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